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Alcohol and Protein Synthesis

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Introduction: Neurological Aspects and Complications of Alcoholism

Stanley van den Noort

Abstract

Neurological concomitants of alcoholism and alcohol intoxication, including pathological intoxication, are described. Symptomatic treatment of alcohol withdrawal is covered, and delirium tremens is discussed in detail. Nutritional deficits in alcoholism and subsequent nutritional and metabolic encephalopathies, alcohol-related myopathies, prenatal neurological damage, trauma and accidents, hypoglycemia, hangover, and conditions resulting from ingestion of alcohol contaminants in "moonshine" are likewise described. The author concludes that alcohol use should be accepted, but calls for abuse prevention efforts, treatment for alcoholics, and use taxes to pay for costs involved.

The basic change that alcohol causes with short-term use remains poorly understood. While ethanol is known to alter membrane and inhibit the ATPase responsible for sustained membrane polarization and function, it is likely that other and diverse effects occur in neuronal, synaptic, glial, and vascular elements. The early intellectual and emotional effects that form the probable basis of social drinking may relate to decreased inhibition, released behavior, and a sense of well-being. The industry of tipping in restaurants and other circumstances is probably dependent on this state. However, responses to alcohol are highly variable in different settings and different individuals and at different levels of maturation. Even with small doses of alcohol, motor, sensory, and coordinative skills decline.

More substantial doses of alcohol produce the altogether familiar picture of the lethargic, slouched person with reddened face and slurred speech, widespread legs, unsteady gait, and clumsy grasp. Neurologists and traffic police demonstrate the loss of cerebellar coordination by testing an individual's ability to walk heel-to-toe and to bring the tip of the index finger from the nose to a distant object. Movements of the eyes in following an object develop the rapid jerking and slower compensatory movements of nystagmus. With the recently intoxicated person lying on his side, the jerking

movement is downward while the blood alcohol is rising and is upward while the blood alcohol is falling. Presumably, this is due to differential alcohol concentrations in the receptors and fluids of the vestibule. The ability to endure trauma without pain is common with acute intoxication and reflects the general and local anesthetic qualities of ethanol. As noted by Shakespeare, autonomic functions are affected most notably with blushing and impotence. The bladder is affected as well. Memory is blunted at low levels and amnesia is common with severe intoxication. At blood levels of 500 mg%, coma ensues and the prognosis is grave without vigorous treatment.

Repeated use of ethanol leads to tolerance through membrane changes that include increased levels of ATPase; alcohol is cleared primarily through hepatic alcohol dehydrogenase, which increases with alcoholism. Metabolites of alcohol such as acetaldehyde are toxic; e.g., the increased degradation of pyridoxal phosphate. Obese patients tolerate more alcohol than thin ones do, and there is wide variation in responses independent of induced tolerance.

Pathological intoxication describes abnormal behavior in response to minor doses of alcohol. I recall an army officer who was a heavy social drinker. A closed head injury with several days of stupor was followed by slow but complete recovery in 2 months. Several years after this injury, modest doses of alcohol would elicit outbursts in which he would smash furniture, steal cars, drive many miles, and resist restraint. Such phenomena may reflect central nervous system disease, but are seen also in individuals without known pathology. Pathological intoxication may occur with other sedatives as well.

Chronic abuse of alcohol leads ultimately to abstinence often triggered by some event: arrest, illness, or injury. Neuronal surfaces and receptors that have become adjusted to the presence of ethanol and its metabolic products become excitable in its absence. It is probable that shifts in pH, magnesium, and other elements play contributory roles. Substantial evidence of magnesium deficit and therapeutic responses to magnesium are difficult to evaluate in view of the known anesthetic properties of the magnesium ion and the multiple associated electrolyte and metabolic changes.

In the first day of withdrawal or reduced intake, there may be "rum fits," usually single, often generalized, sometimes focal, and occasionally repetitive and life threatening. They occur perhaps in some 15 percent of serious alcoholics who are acutely withdrawn from alcohol. Diazepam, magnesium, and carbamazepine may be of

value in treatment and prevention. Each may be of value as a preventive measure in the context of myoclonic jerks, a prior history of seizures, or the prevention of recurrent seizures. Phenothiazines lower seizure threshold and should be avoided in the treatment of alcohol withdrawal.

The tremor, easy startle, anorexia, vomiting, red eyes, wide pupils, fever, and propensity to hallucinosis that characterize alcohol withdrawal may proceed into frank delirium tremens in about 15 percent of people so affected, particularly males. It is hard to improve on the description of Gowers:

> Delirium tremens is far more common in men than in women, the proportion being about six to one. In most cases, the malady follows a severe bout of drinking, often without any withdrawal. Occasionally, there has been a cessation of drinking for a few days before the onset of delirium. The symptoms of delirium tremens usually develop gradually but rapidly, attaining considerable intensity within the course of 2 to 3 days. Disturbed sleep, loss of appetite, and restless irritability are commonly the first indications of the impending disorder. Distressing or horrid dreams of peculiar vividness attend sleep, and the patient cannot shake off their effects when he awakes. He is depressed, uneasy, restless during the day, often annoyed with floating specks or flashes of light before his eyes, and the next night brings the same distressing dreams. As soon as he closes his eyes, unpleasant scenes present themselves before him and he tosses about all night, half awake, or perhaps is unable to contain any real sleep. The next day, from time to time, more definite hallucinations of sight or hearing occur. The patient can shake them off and knows that they are unreal, but they return again and again. When night comes, they take possession of him, prevent all sleep, and the following morning they are no longer to be shaken off. This insomnia, first partial and then absolute, with the increasingly distressing hallucinations which disturb the imperfect sleep that is attained, characterizes the early phase of the disease. The transition to more considerable disturbances is usually attended by unnatural loquacity and by indications of suspicion and uneasiness. The period of the definite delirium begins with the persistence of the hallucinations during the day. They are chiefly visual and at times auditory, and occasionally related to some cutaneous sensations. They are almost always unpleasant. At first, the objects the patient actually sees are transformed into other things than they really are, often into living creatures in active motion. The pattern of wallpaper becomes beetles, spiders, or snakes, and then these appear spontaneously and are crawling about the bed clothes. The sufferer tries to brush them away or to escape from them. And at first, he can be recalled from his aberrations for a few moments and will answer questions correctly, but soon lapses into delirium. Delusions of a wider extent grow out of the hallucinations, or are excited by his bodily discomfort. Faces appear before him, grinning at him, or he fancies that persons are standing by his bedside, upbraiding or abusing him. A characteristic of the delirium is the versatility of false ideas. They change continually. Gradually, he ceases to be able to shake them off, even for a moment, and no longer recognizes his friends or doctor. The attendants are thought to be subjects of the delusions, and he may strike at

them under the impression that they are attempting to injure him. Often, a modified instance of his daily life seems to occur before his eyes. He usually talks incessantly, but may wander incoherently from one subject to another in the course of the same sentence. Auditory hallucinations may occur early or may be grafted on those of vision; the patient thinks he hears reproaches or insults from the fancied bystanders. Less commonly, an unpleasant smell or disagreeable taste annoys him, or he fancies that insects are crawling on his skin. The delusions often excite intense emotions of fear or dread. The characteristic tremor is usually present from the first. It occurs only on movement and is irregular but considerable in range. It is most conspicuous in the arms, the face, and the tongue, but is also seen in the legs when these are put into voluntary movement. It is more conspicuous because the patient usually is in constant movement, kicking his bed clothes, searching for imaginary objects, and attempting to get out of bed. This extreme restlessness is seldom absent. And often, in addition to tremor or movement, there are spontaneous, slight, partial muscular twitchings. And in severe cases, these may amount to considerable, shock-like contractions, and may occur in the muscles of the trunk, as well as in the legs. The countenance of the patient is flushed, but it must be injected, the patient occasionally is pale. The pulse is frequent and soft. The frequency is proportional to the severity of the attack and its duration. At first, the pulse is full and large, but as the disease goes on, it becomes smaller and becomes more frequent. The patient usually perspires freely. The temperature is raised in all but the slightest cases. Occasionally, great fever is attained. The tongue is thickly coated and there is usually complete anorexia. The patient may or may not be thirsty.

This state usually continues for 2, 3, or 4 days without interruption. The patient gets no sleep or only dozes for an hour. The pulse becomes softer and more frequent. In favorable cases, the patient at last falls into a sound sleep, which continues for 8 to 12 hours, and awakes free from all mental disturbance, or with only a trivial amount of delirium, which another sleep entirely removes. The tremulousness often continues in a slight degree, sometimes for days and weeks.

Sustained use of alcohol is regularly attended by inadequate intake of other nutrients because of satiety, the pursuit of alcohol at the expense of food, and the effects of alcohol on gastrointestinal function. Thiamine deficiency is the paramount problem, but deficits of pyridoxine, niacin, other vitamins, and minerals are seen. Pyridoxine deficiency in the adult is usually manifest as a neuropathy and aggravated by drugs commonly used in the alcoholic; e.g., isonicotinic hydrazide. Niacin deficiency affects the nervous system as pellagra, producing the triad of photosensitive dermatitis, diarrhea, and dementia; a rarity today, it may be seen in some malabsorptive states. Pellagra has a rather distinctive neuropathological marker in cerebral chromatolysis.

The primacy of thiamine depletion reflects its multiple roles in the nervous system. Thiamine phosphate is essential for nerve

conduction. It is an essential cofactor in the oxidative decarboxylation of pyruvate and alpha-keto-glutarate in the Kreb's cycle. Thiamine is necessary for transketalase in the pentose-shunt alternative pathway of glucose metabolism. Clinical thiamine depletion is best marked by measuring erythrocyte transketolase and the in vitro response to thiamine phosphate. Thiamine depletion is not limited to alcoholism; it may occur in the general malnutrition most commonly recognized as beri-beri in Asia, where diets of polished rice contain scant thiamine. Curiously, condiments such as garlic may greatly accelerate thiamine absorption.

The most common manifestation of thiamine depletion in alcoholic malnutrition, probably admixed with direct toxic effects of alcohol, is polyneuritis. It is hardly surprising that cells that must extend their metabolic machinery from neck to toe are particularly vulnerable to toxins or deficiency states. In alcoholic polyneuropathy, it is the distal extremities of the nerve fibers that are affected with later changes in the supporting myelin and proximal elements; this is aptly called "dying back" neuropathy. Clinical features are usually more severe in feet than hands, and while affecting both sensation and strength, the sensory changes are usually more prominent. Burning feet and exceptional tenderness to pressure are common. The tenderness is unexpected, but may reflect the general phenomenon of raised threshold for minimal response but exaggerated coarse responses to stimulation above the new threshold. On testing with pin or touch, one encounters a graded loss of distal sensation, so that light touch may be lost below the knee while slightly heavier touch is lost below the ankle. This finding is the hallmark of neuropathy, an important differentiating sign not seen in cord or root disease, and is *not* well described by the term "glove and stocking loss." Weakness of toes and ankles may add to the already increased risk of injury in the alcoholic. The tendon jerk at the ankle is often permanently lost.

Malnourished peripheral nerve is more susceptible to ischemic-pressure injury. Foot drop and wrist drop after heavy motionless sleep under the influence of alcohol commonly occur in the context of thiamine depletion. Similar changes are seen when sleeping pills or pain remedies are given to patients with nerves made more susceptible by diabetes, malnutrition, or neuropathic drugs.

In severe thiamine depletion, neuropathy may be generalized, may affect autonomic nerves, and may set the stage for the remarkable central phenomenon of Wernicke's encephalopathy and its untreated end products of Korsakoff's syndrome or death.

Wernicke's encephalopathy is seen in the malnourished alcoholic admitted for some illness such as pneumonia and dehydration. Intravenous fluids containing glucose rapidly deplete small reserves of thiamine, leading to a state of apathetic confusion, ataxia, nystagmus, and failure of lateral eye movement. Stupor, coma, and death often supervene. Prompt intravenous thiamine in large doses of 100 mg repeated several times a day for several days will dramatically reverse early stages of the syndrome, particularly the opthalmoplagia. Survival with late or inadequate treatment is marked by striking failure of recent memory as a permanent residual. In the early weeks of this amnesic state, patients may substitute remote or reconstructed memories in the form of detailed confabulations for their actual experience. Pathology attending death in the acute phase is marked by edema, hemorrhage, and necrosis affecting the medial thalamus, the periventricular gray matter, and the mamillary bodies. There is often atrophy of the cerebellar midline.

A relatively small number of alcoholics develop a progressive and continuously unsteady broad-based gait resembling the acute effects of alcohol. Truncal instability may be manifest by vertical movements of titubation. This alcoholic cerebellar degeneration may improve slightly with sustained abstinence, but is exaggerated by small amounts of alcohol. To what degree this disorder is due to deficiency, to alcohol toxicity, or to special susceptibility in some alcoholics remains unclear. The common finding of vermal atrophy in patients with Wernicke's encephalopathy favors malnutrition as an important factor.

Folic acid deficiency is very common in the alcoholic and manifest as a macrocytic anemia. The evidence that folate deficiency contributes to neurological dysfunction in alcoholism remains unconvincing.

Alcoholics who smoke heavily and thiamine-depleted war prisoners may sustain subacute substantial loss of central vision that improves to a degree with nutritional supplementation. Many of the neurological complications of alcoholism are less clearly linked to dietary deficiency. There is increasing recognition of the sustained intellectual decline and the brain atrophy in a majority of chronic alcoholics. This cannot be regularly ascribed to malnutrition or to trauma.

Some evidence of zinc deficiency has been reported, but it is not likely that zinc will protect the brain of the alcoholic from intellectual decline.

An unusual complication of alcoholism also seen in other metabolic encephalopathies is central pontine myelinolysis with stupor, quadraparesis, and bulbar dysfunction. About half of these cases occur in alcoholics, and the concomitant pathology of Wernicke's encephalopathy is seen in about one-quarter of these cases. Similar striking necrosis of central pontine structures has been demonstrated in hyponatremic animals subjected to rapid infusion of saline. The anatomic arrangement of myelinated tracts crossing through neuronal clusters appears to favor disruption under these circumstances. Most cases of central pontine myelolysis occur in the context of hyponatremia and its correction, arguing strongly for cautious increments of salt replacement in the treatment of hyponatremia.

Most mysterious of all and quite befitting its euphonious eponym is the Marchiafava Bignami syndrome. Presenting as a nonspecific dementia with or without seizures, its striking pathology is most difficult to explain. Sharply demarcated extensive necrosis of the rostral midline of the corpus callosum is the hallmark of this disorder. In some patients, necrosis may also be seen in the anterior commisure, centrum semiovale, optic chiasm, and cerebral peduncles.

Acute and chronic forms of myopathy round out these ill-defined effects of alcohol. Abrupt forms of proximal weakness with muscle necrosis, sometimes preceded by documented hypokalemia, or more indolent forms of proximal weakness and wasting in shoulders and thighs are uncommon, but are regularly seen in medical centers that serve populations of severe alcoholics.

The neurological carnage of chronic alcoholism extends across the placenta, producing a syndrome of retardation, small stature, microcephaly, and small central facial features in the fetus.

Laennec's cirrhosis in alcoholism may have several affects on neurological function. The cirrhotic with gastrointestinal hemorrhage absorbing an unmanageable load of nitrogen with attendant hyperammonemia develops lethargy and a curious defect in sustaining the posture of the extended wrist. This "flap" is not specific for hepatic failure and may be seen in drug intoxications and other metabolic disorders. Lethargy proceeds to stupor, and large rostral slow waves appear in the electroencephalogram. A few patients, particularly those with surgical bypass of the portal circulation, develop a fluctuating recurrent confusional state with confusion, ataxia dystonic postures, and choreic movements; this may respond to dietary restriction of nitrogen and the use of antibiotics to

deplete intestinal flora. Cirrhotics may also have parenchymal brain hemorrhage in sites such as caudal centrum semiovale, which are not usually affected by brain hemorrhage in other diseases; deranged clotting mechanisms and impaired liver function may be important factors in such cases.

Perhaps the most common neurological and general complication of alcohol abuse is trauma. Alcohol-related automobile accidents kill more than 50 people in the United States every day. The toll of other forms of accidents and nonfatal injuries and the damage to those innocently involved is enormous. It would be very difficult to get an accurate estimate, but it would be mine that at least 20 percent of the medical costs in the United States ($40 billion), or 2 percent of our gross national product, is directly or indirectly a consequence of alcohol abuse. A portion of these costs derive from all of the aforementioned effects of alcohol. An additional factor is the reduced ability of the alcoholic to withstand infection. In our society, adult pneumonia, tuberculosis, and diphtheria are seen predominantly in the alcoholic.

A small but important neurological aspect of alcoholism is its relationship to essential tremor. This is a tremor of hands, voice, and/or head, most commonly manifest in active use of the hand in its midposition as in writing or holding a cup. It appears at adolescence or later in life, with a probability that a parent was also affected. The tremor is exaggerated by stress and fatigue; it is alleviated transiently but quite dramatically by alcohol. Subjects with essential tremor may become alcoholic in affecting control of their tremor just as some schizophrenics will "control" their thought disorder with alcohol; in both cases, the long-term effect of alcohol therapy usually makes the underlying disease worse and superimposes the complications of alcohol abuse.

Alcoholics and perhaps some heavy social drinkers become subject to delayed hypoglycemia. In the latter group, this may be manifest as functional hypoglycemia without disability, but in the former, it can produce coma and death if not recognized and treated. This is due to decreased gluconeogenesis during inebriation.

The ubiquitous neurological complication of alcohol is the hangover, which needs no description to most readers and remains a mystery in its mechanism. Laboratory studies of ingested alcohol usually do not provoke hangover. The role of various cogeners, tyramine in wine, smoke, conversation, and guilt seems as important as the alcohol itself. On occasion, one may encounter an

individual who seeks medical attention for headache who is having delayed hangovers, which may appear 16 hours or more after the cessation of drinking. Migraineurs are often very sensitive to alcohol. A trial of abstinence may be of value in the management of frequent headache.

Finally, one must consider the contaminants of alcohol that cause neurological problems. Methanol can lead to blindness in small doses. Individuals vary widely in sensitivity to methanol. Methanol with ethanol is better tolerated, and indeed intravenous ethanol is the treatment of choice for methanol poisoning. The manufacture of "moonshine" has many hazards, not the least of which is collusion between treasury agents and hardware merchants who sell copper pipe. In response, resourceful distillers abandoned copper coil for old automobile radiators with holes plugged with lead solder. Lead neuropathy in "moonshine" drinkers is a recurrent problem in the surrounds of Appalachia. A most effective organic solvent with many industrial uses is triorthocresyl phosphate (TOCP). During Prohibition, TOCP-contaminated alcohol (often sold as Jamaica Ginger) caused many cases of "Jake paralysis." This provided a delayed onset of a polyneuritis, then a slow recovery over many months, only to uncover spastic paraplegia due to a concomitant myelopathy.

It is clear that alcohol has done many bad things to the human brain and to human behavior over the several millennia of its use and abuse. It continues to pose a serious problem. But on the other side, it has been a source of human solace and comfort to which many people turn to modify the pains of life and to enhance its pleasures. Something that has claimed our attention and devotion for six or more millennia is likely to have more potential for human comfort than the converse. Until the 19th century, it was also our major anesthetic. A society without alcohol may be a better society, but it may also be a more violent one and a less happy one, or it may turn to alternative agents such as marijuana, which, given the license of ethanol, may pose medical and social problems of equal or greater magnitude. I personally believe that we should accept the use of alcohol, try to prevent its abuse, try to cure those who have abused it, tax all users to help pay for the cost of abuse, and propose an occasional toast to this particular resource for human comfort.

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GROUP 1: ALCOHOL AND MECHANISM OF BRAIN MACROMOLECULAR SYNTHESIS

The Effects of Alcohol on the In Vivo Incorporation of Amino Acids in Mouse Brain

Eugene Toth and Abel Lajtha

Abstract

We tested the influence of alcohol on protein synthesis in the mouse brain in vivo by administering alcohol acutely, in a single dose, or chronically, over many days or months, and by giving alcohol to pregnant females. In each case, the incorporation of a loading dose of (^{14}C)valine was measured over a 2-hour period. In acute experiments, ethanol was administered intragastrically (30 percent solution). Up to a 4 mg/g dose, alcohol had no effect; 8 mg/g decreased the amino acid incorporation into brain proteins. The inhibition was 56 percent when the animals were kept at room temperature (their body temperature dropped to 30°C) and 18 percent when they were kept at 30°C (body temperature stayed at 38°C). In chronic experiments, we tested incorporation after 14 days of alcohol treatment. After mice were kept on a liquid diet containing 7 percent ethanol for 2 weeks, incorporation (for 2 hours) was measured in whole brain and in subcellular fractions. The results were similar in Swiss mice (3 weeks old at the start of the experiment) and in C57BL/6 mice (6 weeks old at the start): protein synthesis in whole brain decreased about 6 percent, in microsomes and cytosol about 12 percent, and in synaptosomes over 20 percent. For longer experiments, Swiss mice were kept on drinking water with 10 percent ethanol for 7 weeks. The decrease in incorporation was about 10 percent in whole brain, 6 percent in the cytosol, and 12 percent in synaptosomes. Maternal ethanol consumption (mothers consumed drinking water with 10 percent ethanol for 3 months prior to conception and during gestation) decreased the rate of protein synthesis in the 20-day-old fetus by about 10 percent; brain DNA per gram of tissue was also similarly decreased. The rate of incorporation of neonates (20 to 72 hours

NOTE: Tables appear at end of paper.

old) was normal, and DNA content returned to normal after 72 hours. We conclude that small (acute) doses of alcohol in short-term experiments do not affect brain protein synthesis, whereas large (acute) doses (even if body temperature is maintained) and chronic long-term alcohol ingestion decrease protein synthesis by 10 to 18 percent.

It is now generally agreed that the turnover of proteins proceeds at relatively high rates in the brain. There is a consensus among various laboratories on the value of average rates of incorporation of amino acids into proteins of young and adult brain (Dunlop, Lajtha, and Toth 1977). Although metabolic rates under physiological conditions are well established, changes observed under a variety of other conditions are not as generally accepted (Lajtha et al. 1979). Brain protein metabolism seems to be relatively stable, but it is altered by a number of factors, often in a specific manner. An example of such stability and relative specificity is the effect of low-protein diet on cerebral protein turnover. Protein metabolism in adult brain is not affected by even prolonged protein malnutrition, but in other organs it is seriously affected. In the rapidly growing phase, protein turnover in brain is inhibited—but differently from that in most other organs in that both synthesis and breakdown are inhibited (Banay-Schwartz et al. 1979), resulting in a smaller change.

Protein Turnover Rates In Vivo

It is likely that most, and possibly all, proteins are in a dynamic state, undergoing synthesis and breakdown many times throughout the life of the brain and thus being vulnerable to factors that alter metabolism. Although incorporation has been measured in several studies, only a few have tried to determine whether there are stable proteins in brain. In an earlier study (Lajtha and Toth 1966), mice were fed a diet containing labeled amino acids throughout gestation and later growth to label all the proteins. When the animals became adult, the diet was changed to an unlabeled one; with time, most of the radioactivity disappeared from the brain, indicating that the stable component may be negligible (table 1). The fact that the rate of disappearance was fairly steady indicates that any pool with a much lower than average turnover rate would have to be rather small.

This method (release of previously incorporated label) gives only lower limits of incorporation rates; because of local reutilization of

the labeled amino acid released, turnover is likely to be underestimated. In one study we calculated the rate of turnover from incorporation as more than twice as high as that calculated from the rate of decrease of label.

We measured turnover rates after extending the time of incorporation to 5 days; by this time, about one-third of brain proteins were replaced (Lajtha et al. 1976). We reasoned that labeling for a longer time would enable us to detect any major slowly metabolized pool that might not be detected in shorter experiments, where usually less than 2 percent of the protein is labeled. The rate of incorporation decreased with increasing experimental time (table 2), indicating that metabolic rates were heterogeneous. Although a more detailed kinetic analysis probably would identify more fractions, with different turnover rates, the first approximation indicated only a small, rapidly metabolized fraction (4.5 percent of the total, with a half-life of about 10 hours) and a large, more slowly metabolized fraction (95 percent of the total with a half-life under 10 days.

A similar experiment with developing brain did not show a significant decrease of the incorporation rate with time (Lajtha et al. 1979). The calculated rates were lower than in the fast metabolic pool but higher than in the slow one in the adult brain. This finding would indicate that most proteins have a higher metabolic rate in the immature brain.

A study of the metabolism of myelin proteins (Lajtha et al. 1977) indicated that metabolic rates of each myelin protein are heterogeneous and possibly depend on the location in the myelin sheath. If this is true for other proteins as well, then metabolic rates may also depend on the location of the various proteins; that is, the turnover of an enzyme in neurons may be different from that in glia or different in various brain areas.

The above studies measured amino acid incorporation; that is, protein synthesis rates. Protein breakdown was measured in only a few experiments, but it seems, like synthesis, to be heterogeneous. Our measurements of breakdown of cerebral proteins indicate a higher rate of protein breakdown in young brain than in adult brain (Dunlop et al. 1978); that is, during the rapid growth phase and protein deposition, the breakdown rate is also higher.

Alterations of Cerebral Protein Turnover

Although protein synthesis and changes in synthesis rates during development have been studied in detail and are well established, changes in protein metabolism are less well established. Several report are available showing changes in incorporation of amino acids into brain proteins under a variety of conditions, but in most cases the experimental approach was such that it is difficult to distinguish effects on protein turnover from effects on the pools, uptake, and metabolism of the administered precursor amino acid. The discussion of such studies is beyond the scope of this chapter, but even with the difficulty of arriving at conclusions from many of the studies (especially the early ones), there is now evidence that the rate of protein metabolism is altered under a number of conditions.

Recently, we studied the effect of malnutrition caused by low-protein diet on brain protein metabolism—a problem that has been investigated in several laboratories. Previous work (Zamenhof et al. 1971) showed that the young brain is vulnerable, whereas the adult brain is resistant, to the effects of malnutrition. The younger the animal and the longer and more severe the malnutrition, the greater the decrease in brain protein synthesis. We found that changes in amino acid transport could not be responsible for this difference in sensitivity between immature and adult brain protein metabolism (Toth and Lajtha 1980). In severe malnutrition in the young, brain protein metabolism changes were somewhat different from changes in other organs in that not only synthesis but also breakdown was decreased (Banay-Schwartz et al. 1979).

Effect of Alcohol on Amino Acid Incorporation In Vivo

Several laboratories have investigated the effect of alcohol on protein synthesis in the brain in vivo and in vitro. The in vitro systems were generally more sensitive to the effects of alcohol (Choy et al. 1972; Morland and Sjetnan 1976; Noble and Tewari 1973). A study of subcellular fractions found no effect from chronic alcohol treatment, but in acute experiments incorporation in microsomes (but not mitochondria) was inhibited (Renis et al. 1975). Although these studies showed an inhibition of brain protein

synthesis, tracer doses of leucine were used, and under such experimental conditions the amino acid was probably rapidly metabolized and its specific activity rapidly changed with time. We decided to confirm these studies with experiments in which the metabolism and specific activity changes of the precursor amino acid are minimal.

Methods

We studied incorporation in male Swiss mice bred in our laboratories. When the animals received the regular diet, alcohol was administered intragastrically for acute experiments or was added to the drinking water in chronic experiments. In some experiments, mice (C57B1/6) from an alcohol-preferring strain (Kakihana et al. 1966) were kept on a liquid diet (Carnation Slender, Carnation Co., Los Angeles, Calif.) containing ethanol (experimental) or sucrose (control).

For measuring incorporation, a large "flooding" dose of (^{14}C)valine was injected intraperitoneally. Such a dose does not alter the rate of protein metabolism, but by increasing cerebral valine levels severalfold, it maintains the specific activity of free valine in the brain at a level close to that in the injected solution for more than 2 hours (Dunlop et al. 1975*a*). Maintaining the specific activity throughout the experiments and in all experimental animals permits comparison of experiments with various conditions and calculation of incorporation rates in each case.

Effects on Proteins in Adult Brain

Low doses of alcohol did not have significant effects on brain protein synthesis. When the dose was increased, the incorporation of valine was inhibited (table 3). With large doses of alcohol in acute experiments, the decrease was very large (56 percent); however, this decrease was partially due to the hypothermia caused by the alcohol administration. In previous studies (Dunlop et al. 1975*b*; Lajtha and Sershen 1975), amino acid incorporation in brain was found to be dependent on the temperature of the organ, and the rate was found to decrease approximately 7 to 8 percent per 1°C decrease of temperature. When the experiments were repeated

with the temperature of the animals kept at control levels (by keeping them in an incubator at 34°C), the effect of alcohol was less but still significant (table 3). Under chronic alcohol administration, when body temperature was maintained, incorporation was inhibited to a moderate but significant degree (about 10 percent). There was a tendency (statistically not significant) toward increased incorporation in the withdrawn animal.

The small inhibition of incorporation into total proteins is an average value; specific fractions may be inhibited more or less than average. Under chronic conditions, the largest decrease in incorporation occurred in the synaptosomal fraction: the rate of turnover in this fraction was lower than in other fractions, but the degree of inhibition of incorporation was greater (table 4). When the brain was divided into four large areas, the regional differences in the inhibition of incorporation were found to be not significant. It must be emphasized that under our conditions the inhibition was rather small (10 percent). The observed changes in each fraction indicate that the effect of alcohol is general, involving many rather than a few specific proteins.

Effects on the Developing Brain

Because developing brain in general is more sensitive to outside influences, it was of interest to test the effect of alcohol on the incorporation of amino acids in immature brain. Under our experimental circumstances (putting the mothers on water containing 10 percent ethanol throughout pregnancy and lactation), incorporation was inhibited in the immature brain to the same degree as that in adults. In the 20-day-old fetus, the inhibition tended to be slightly greater (table 5). Testing of various fractions in the 5-day-old mice showed that incorporation into synaptosomes and nuclei was inhibited more than into soluble proteins, although the turnover rate was higher in the soluble fraction. Incorporation into myelin proteins was slightly increased in the alcohol-treated animals. In the young brain, the rate of incorporation, as in previous studies, was 2.3 to 3.6 times higher than in the adults.

The Effect of Alcohol on the Growth of Brain

In a recent study, Smith-Kielland and Morland (1979) found no changes in protein content or organ weight of adult brain after alcohol treatment. Putting the mothers on drinking water containing alcohol for a prolonged period resulted in a decreased body weight in the newborn animals (table 6). Body weight in the first few days after birth was about 20 percent lower than in the control group. Brain weight was also significantly decreased; the decrease in brain weight was greater than the decrease in brain proteins. It is possible that the water content of the body and the brain is decreased in alcohol-treated animals, resulting in a greater change in tissue weight than in protein content. Although DNA content also tended to be decreased, this change was not significant (table 6). The developing brain is more sensitive than the adult organ to alcohol, as shown by changes in protein content and organ weight. Similar differences were observed in protein content and organ weight changes in young and adult brains during malnutrition.

Possible Changes in Protein Breakdown

Although the difference is not statistically significant, a comparison of decrease in incorporation in the immature brain (table 5) with the decrease in brain protein (table 6) shows a tendency for greater decrease in incorporation than in protein content. We did not measure changes in protein breakdown, but a difference in breakdown should be expected from the greater change in incorporation than in protein content, indicating that alcohol inhibits both protein synthesis and protein breakdown.

In our study of the effects of low-protein diet on brain protein metabolism (Banay-Schwartz et al. 1979) (in which we found that malnutrition caused a decrease of both synthesis and breakdown of protein, with the decrease in breakdown less than in synthesis, resulting in decreased protein content), the decrease in protein breakdown under conditions of decreased synthesis may have been a mechanism to preserve brain proteins. In this respect, protein metabolism in brain can be different from that in organs such as muscle and liver, which under some circumstances supply amino acids to the organism through an increase of protein breakdown

during a decrease of synthesis. Measurements of effects of alcohol on the breakdown of proteins are in progress in our laboratory.

Conclusions

The effect of alcohol on the mechanism of protein synthesis in isolated systems has been shown clearly and in some detail in an elegant set of experiments by Tewari and Meeker (see their most recent review, this volume). The effects of alcohol on protein metabolism of the brain are less in the living animal than in in vitro; in vivo, small doses of alcohol do not have any effect on brain protein synthesis, and large doses (considerably larger than human alcohol consumption) have a small but significant inhibitor effect (under our conditions, about 10 percent inhibition). This inhibition is slightly larger in the immature brain. A crude fractionation into four brain areas did not show significant regional variations, but the somewhat greater decrease of incorporation in nuclear and synaptosomal fractions and the increased incorporation into myelin proteins indicate some differences in alcohol effects on the metabolism of specific proteins. The rapidly metabolizing proteins are not more sensitive to incorporation, suggesting that protein breakdown is also inhibited by alcohol. Whether this protective mechanism, the decreasing of breakdown, involves all or only some brain proteins is not established at present.

Table 1. Stability of Proteins in Brain

Experimental Time (days)[a]	Cts/Min/μmol Lysine in Protein	Percent of Original Specific Activity Remaining
0	30.0	100.0
30	8.8	29.0
60	2.1	7.0
150	0.7	2.4

SOURCE: Lajtha and Toth 1966. Reprinted from *Biochem Biophys Res Commun,* 23:294-298, 1966, by permission. Copyright 1966 by Academic Press.

[a] 0 = 60-day-old Swiss mice just before the labeled diet was replaced with the unlabeled diet. All brain proteins were labeled in this experiment, and most of the label was replaced by 150 days.

Table 2. Turnover of Brain Proteins in Long-Term Incorporation Experiments

Incorporation Time (hours)	Protein Specific Activity (percent of free)	Turnover in Time Interval (percent/hour)
0	0.0	
		0.66
1	0.6	
		0.51
10	5.1	
		0.42
24	10.0	
		0.34
50	17.0	
		0.28
120	33.0	

SOURCE: Lajtha et al. 1976. Reprinted from *Biochim Biophys Acta,* 425:511-520, 1976, by permission. Copyright 1976 by Elsevier Biomedical Press B.V.

Note: The specific activity of the precursor amino acid was kept constant throughout the experiment. The rate of incorporation decreased with time, showing heterogeneity of turnover rates.

Table 3. Effect of Alcohol on Amino Acid Incorporation in Adult Mouse Brain in Vivo

Experiment	Body Temperature	μmol Valine per g Brain	Cts/min/mg Protein	Percentage Decrease
Control	38.2	2.0	140	–
Chronic alcohol	37.4	2.7	127	9
Withdrawn 15 hours	38.4	2.4	147	0
Acute alcohol, high dose	30.1	2.8	62	56
Acute alcohol, at 34°C	39.3	1.8	115	18
Acute alcohol, low dose	38.2	2.4	132	6

Note : Incorporation of (^{14}C)valine was measured after 2 hours (15 μmol/g body wt, injected intraperitoneally). The level of valine in the brain without valine injection is 0.12 μmol/g; the level during incorporation of about 2.4 represents a twentyfold increase. In acute experiments, ethanol was given intragastrically (8 mg/g, high dose; 3 mg/g, low dose) 30 minutes before valine to adult mice in a 30 percent solution. In chronic experiments, animals were given alcohol (10 percent) instead of drinking water for 20 weeks. Averages of five experiments are shown, with a standard deviation within 6 percent of the mean.

Table 4. Effect of Chronic Ethanol on Incorporation Into Protein Fractions

Fraction	Incorporation	
	Cts/min/mg	Percentage of Control
Total protein	186	90
Soluble protein	210	85
Insoluble protein	162	95
Synaptosomes	73	80
Mitochondria	134	94
Endoplasmic reticulum	331	96
Cerebellum	212	89
Thalamus	170	92
Pons medulla	165	91
Cortex	204	90

Note: The conditions were the same as in the experiments described in table 3. Incorporation was measured 2 hours after a flooding dose of (^{14}C)valine. Chronic treatment was substitution of 10 percent ethanol for drinking water for 20 weeks. Averages of four experiments are given, with a standard deviation of 6 percent of the mean.

Table 5. Effect of Chronic Maternal Alcohol Intake on Cerebral Amino Acid Incorporation in the Offspring

Experiment	Brain Valine (μmol/g)	Incorporation (Cts/min/mg)	Percentage of Control
Mother	1.8	196	92
Fetus, 20 days old	5.5	706	83
Newborn, 1 day old	4.5	447	86
3 days old	3.7	450	89
5 days myelin		339	115
Synaptosomes		313	96
Nuclei		290	91
Cytosol		520	97
Particulate		474	91

Note: Mothers were given 10 percent ethanol instead of drinking water for 14 weeks before and during pregnancy and lactation. Two-hour incorporation of a flooding dose of (^{14}C)valine was measured. Averages of six experiments are given, with a standard deviation of 5 percent of the mean.

Table 6. Effects of Chronic Alcohol Treatment in Young Mice

	1 Day Old		3 Days Old	
	Control	Percentage of Control	Control	Percentage of Control
Body weight	2.10 g	83	3.24 g	80
Brain weight	106.00 mg	86	150.00 mg	86
Brain protein	5.83 mg	95	8.40 mg	93
Brain DNA		96		97

Note: Averages of eight experiments are given, with a standard deviation of 7 percent of the mean.

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Effect of Ethanol and Ethanol-Induced Hypothermia on In Vivo Net Protein Synthesis in Fetal Rats and Placenta*

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Abstract

The effects of acute and chronic maternal ethanol intake and concomitant hypothermia on fetal net protein synthesis (NPS) and on placental transfer of amino acids were investigated. NPS rates were determined by the rate of incorporation of (^{14}C)valine (500 mM, SC) into total extracted protein. Acute maternal ethanol exposure for 2 hours (4 g/kg, po) on day 20 of gestation resulted in hypothermia (35.7°C) and in depressed ($p < 0.05$) NPS in fetal brain, heart, kidney, liver, and placenta (20 to 25 percent reduction). When maternal hypothermia was prevented, only fetal liver NPS remained depressed ($p < 0.05$). Chronic ethanol exposure per se had no effect on fetal or placental NPS unless maternal blood ethanol levels were elevated by a loading dose of ethanol (4 g/kg, po). This reduced NPS in placenta and in fetal brain, heart, kidney, and liver by 20 percent, 17 percent, 8 percent, 17 percent, and 20 percent, respectively ($p < 0.05$). Normalizing maternal body temperature reversed NPS reductions in fetal brain and kidneys ($p > 0.05$). Both chronic and acute ethanol regimens were also found to reduce ($p < 0.05$) placental transport of valine (Val, 10^{-6}M) by 40 to 44 percent independent of ethanol-induced hypothermia. Placental transport was determined in vivo following intravenous injections of valine and in vitro in placental villous fragments. Two-hour in vitro treatment of previously unexposed villous fragments to ethanol (1 to 3 mg/ml) reduced ($p < 0.05$) valine

NOTE: Figures and table appear at end of paper.

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uptake by 26 to 36 percent, where at least 460 μM acetaldehyde was required to elicit an effect. It is concluded that (a) both ethanol-induced hypothermia and ethanol per se depress NPS in vivo in placenta and in fetal rats, (b) chronic and acute maternal ethanol consumption reduces placental valine uptake, (c) ethanol is probably the causal factor in the latter, and (d) altered placental amino acid transport and reduced fetal NPS may be two mechanisms by which maternal ethanol intake induces fetal growth deficits.

Introduction

Fetal alcohol syndrome (FAS) in humans is represented by a specific pattern of craniofacial, developmental, and structural abnormalities seen in offspring of women who have consumed alcohol heavily during pregnancy (Jones et al. 1973; Lemoine et al. 1968). The most commonly seen abnormalities are the typical facial characteristics, motor dysfunction, impaired intellectual function, and pre- and postnatal growth deficiencies (Hanson et al. 1976; Jones et al. 1973). The cause or causes of this syndrome have not yet been identified, but they do not appear to be due to a generalized maternal undernutrition. Practically all children diagnosed as FAS show pre- and postnatal growth deficiency with no catch-up phase later in life (Hanson et al. 1976), even in the presence of adequate nutrition.

Studies with animals have demonstrated that maternal ethanol intake induces increased fetal and neonatal mortality (Henderson and Schenker 1977; Henderson et al. 1979; Kronick 1976), and reduced weight of the offspring (Abel and Dintcheff 1978; Henderson et al. 1979; Schwetz et al. 1978). The following studies were designed to quantitate these two developmental abnormalities and to investigate one mechanism by which they might have been induced.

Effect of Maternal Ethanol Consumption on Fetal Growth and Viability

The effects of three ethanol regimens on prenatal growth and fetal viability were investigated (Henderson et al. 1979). Sprague-Dawley rats were administered ethanol perorally (po) (25 percent solution, 5 g/kg) twice a day for 3 consecutive days (days 11 through 13 and 14 through 16) during gestation. Other rats were maintained on the Lieber-DeCarli liquid diet containing either 6 percent

ethanol (v/v) or isocalorically balanced maltose-dextrins for at least 30 days prior to and throughout gestation. Dams were sacrificed on day 20 of gestation. Table 1 indicates that all three regimens cause striking increases in fetal death and a significant ($p < 0.05$) 20 percent decrease in fetal weights. This decrease in fetal weights is accompanied by reduced fetal brain and viscera weights (figure 1A). The most extreme reductions in fetal organ weights were found in the kidneys (24 to 40 percent), whereas brain weights were the least affected (no more than 16 percent). If these data are expressed as a function of fetal body weights (figure 1B), only the kidney/body weight ratios in chronically exposed fetuses and in fetuses with acute exposure on days 11 through 13 remained significantly ($p < 0.05$) reduced. Fetal brain weights following acute exposure decreased to a lesser degree than did body weight, indicating a brain "sparing" phenomenon. Although not illustrated in figure 1, placental weights were altered by chronic ethanol exposure. Mean values for 27 pairs of pregnancies showed that individual placental weights were 0.50 gm $\pm$ 0.01 SE for ethanol exposed versus 0.44 gm $\pm$ 0.01 SE for pair-fed controls—a significant ($p < 0.05$) 12 percent increase.

As indicated above, acute and chronic maternal ethanol consumption can induce significant reductions in prenatal growth in the rat. Other animal studies have shown both this and a postnatal growth deficiency resulting from in utero ethanol exposure (Abel and Dintcheff 1978; Henderson and Schenker 1977; Schwetz et al. 1978). Thus, ethanol administration to pregnant rodents can induce the pre- and postnatal growth deficits similar to those seen in human FAS, providing us with systems to study the mechanistic basis of the protein is prerequisite for its growth, and because ethanol has been shown to alter protein synthetic machinery (Jarlstedt and Hamberger 1972; Rawat 1976; Tewari and Noble 1971), the following studies were undertaken.

Effect of Maternal Ethanol Consumption on Fetal Net Protein Synthesis In Vivo

Methods

Two animal models were utilized (Henderson et al. 1980). For the studies in which the effects of a single dose of ethanol on net

protein synthesis (NPS) were to be determined, pregnant dams received ethanol (po, 4 g/kg) on day 20 of gestation. This single dose was administered either to dams previously maintained on the chronic regimen or to rats with no prior exposure to ethanol. Rats whose body temperature was maintained at 38°C following ethanol administration were kept in a 34°C incubator throughout the experiment. The method of Dunlop et al. (1975) was used to estimate NPS in vivo. (^{14}C)Valine (10 μmol/g rat, 5 μCi/mmol) was administered subcutaneously (sc) 2 hours following peroral ethanol.

Experimental Data

Effect of Chronic Maternal Ethanol Consumption in the Absence of Hypothermia on Fetal Net Protein Synthesis

The effect of chronic maternal ethanol consumption on NPS in fetal brain, heart, kidney, liver, and placenta is shown in figure 2. No consistent significant difference was apparent between ethanol exposed and control values, minor exceptions being the 0.5- and 2.0-hour time points for the placenta. All incorporation values were linear through the 1.0-hour time point. The dams in this series of experiments were tested prior to their morning feeding, had normal (38°C) body temperatures, and had blood ethanol levels averaging 0.76 mg/ml.

Net Protein Synthesis in Fetal Tissue Chronically Exposed to Ethanol and a Single Loading Dose of Ethanol

Ethanol is known to induce a dose-dependent decrease in body temperature in rodents (Ferko and Bobyock 1978; Freund 1973; Ritzmann and Tabakoff 1976), and hypothermia per se has been shown to reduce NPS in young rat and mouse brain (Dunlop et al. 1976; Lajtha et al. 1979). In light of the above factors and the possibility that blood ethanol levels in excess of 0.76 mg/ml might exert a direct effect on the protein synthesis machinery, the effects of elevated blood ethanol levels and ethanol-induced hypothermia on fetal NPS were investigated. Dams maintained on the chronic ethanol diet as previously described were administered ethanol po (4 g/kg) 2 hours prior to injection of (^{14}C)valine. Their body temperature was reduced by 2 to 2.5°C, and blood ethanol levels were increased to 3.0 mg/ml (figure 3, chronic load). In some experiments, maternal body temperature was maintained at 38°C

throughout the study. As shown in figure 4, NPS in fetal and placental tissues obtained from hypothermic dams was significantly reduced ($p<0.05$) in all cases (open bars). If maternal body temperature is kept at 38°C, NPS is reversed to normal values in fetal brain and kidney but remains depressed in heart, liver, and placenta (stippled bars).

Effects of a Single Dose of Ethanol and Ethanol-Induced Hypothermia on In Vivo Net Protein Synthesis

Figure 5 indicates that prior chronic exposure to ethanol is not prerequisite to an ethanol-induced reduction in fetal NPS. The rats received a single po dose (4 g/kg) on day 20 of gestation followed 2 hours later by (^{14}C)valine injection. Incorporation of label was depressed in all hypothermic tissue (open bars). Normalization of maternal body temperature reversed the depressed NPS to control values in brain, heart, kidney, and placenta but not in liver (stippled bars).

Discussion of Experimental Results

Effect of Maternal Ethanol Consumption on Fetal Growth

Prior studies in this laboratory (Henderson and Schenker 1977; Henderson et al. 1979) have shown that chronic maternal ethanol consumption in rats causes pre- and postnatal growth deficiencies and an increased incidence of fetal death. Acute ethanol exposure for 3-day periods during gestation (days 11 through 13 and 14 through 16) was also found to cause decreased fetal survival and body weights. The latter model was designed to approximate "binge" drinking in the human and implies that this type of maternal ethanol consumption in the human could put fetal development at risk.

Depressed fetal body weights were consistently paralleled by significant ($p<0.05$) reductions in fetal organ weights. Kidney weights were the most sensitive to ethanol exposure, with weight decreases between 32 and 39 percent for chronic and day 11-13 acute rats. In most cases, organ weights decreased in proportion to body weight. The two exceptions were (a) brain weight in the two acute regimens, in which the brain appeared to be spared; and (b) the kidney, which lost mass in significant excess of body weight in acute day 11-13 and chronic fetuses. Kidneys in these two regimens

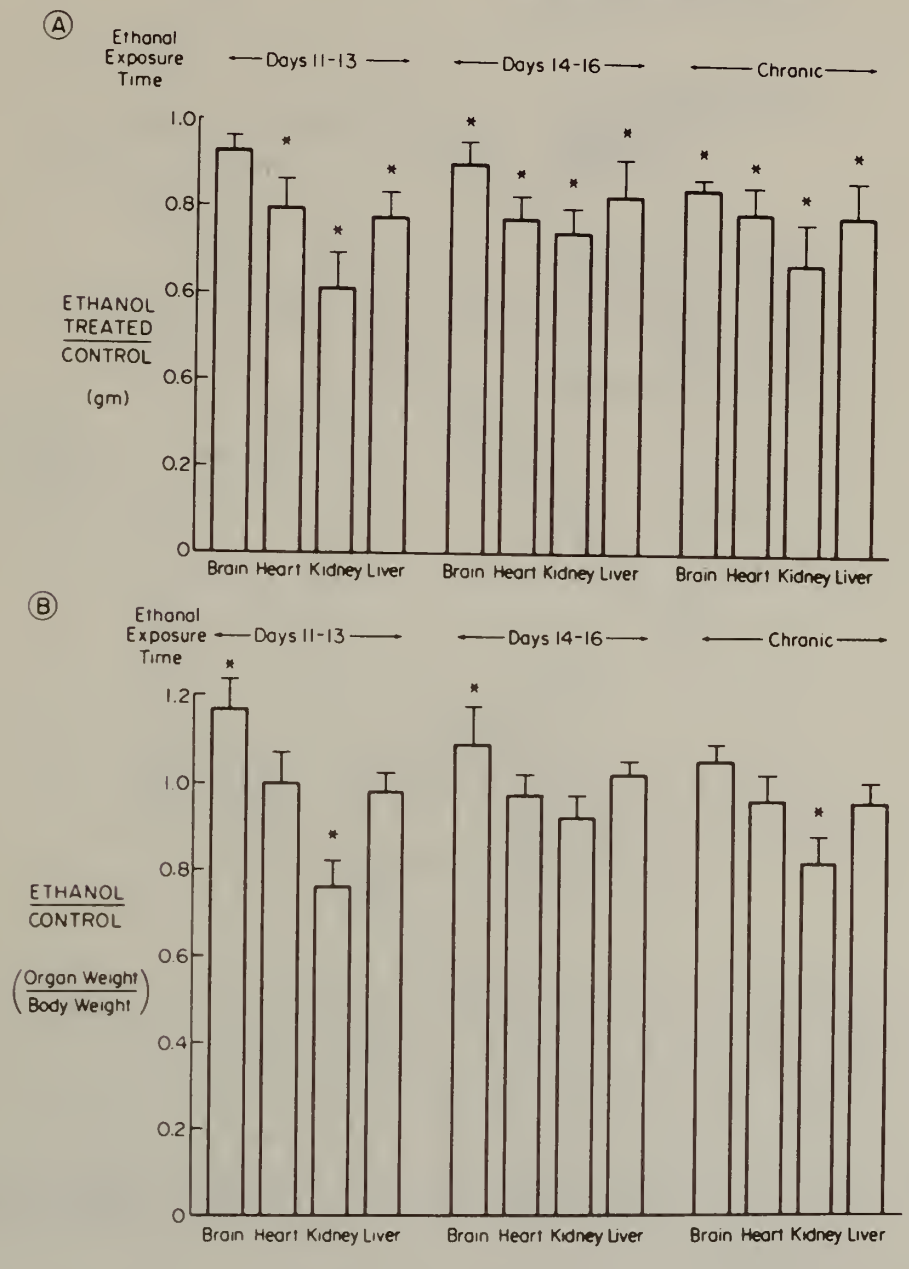
were exposed to ethanol during the time of kidney genesis. The latter results correlate with reports of renal abnormalities in FAS (DeBeukelaer and Randall 1977) and with urogenital abnormalities seen in rodents exposed to ethanol in utero (Boggan et al. 1979).

Effect of Ethanol on Fetal Net Protein Synthesis In Vivo

The preceding studies demonstrate that, in the rat, hypothermia concomitant with ethanol intake plays an important role in the observed reductions in in vivo NPS in fetal and placental tissues. A single dose of ethanol (4 g/kg) administered to rats either with no prior ethanol exposure or during a chronic ethanol regimen reduces maternal body temperature by 2.0 to 2.5°C (figure 3). The resulting reductions in NPS in fetal brain and kidney can be completely reversed by maintaining maternal body temperature at a normal 38°C. The same reversal can be seen in placenta and in heart in rats with no prior ethanol exposure. Thus, ethanol-induced hypothermia is the major component of the observed reduction in NPS in vivo in these tissues. However, normalizing maternal body temperature had no reversing effect on the reduction of NPS in fetal liver in both regimens or in placenta or fetal heart of chronically exposed fetuses. Direct effects of ethanol or its metabolite, acetaldehyde, may be the primary factors contributing to the observed depressions in NPS in these tissues.

In conclusion, acute and chronic in utero ethanol exposure can significantly inhibit fetal body, brain, and visceral growth. The mechanism of this fetotoxic effect of ethanol has not been clearly established, but it could be related to an inhibitory action on the protein synthesis machinery. A single dose of ethanol can inhibit NPS in vivo in fetal tissue, and ethanol-induced hypothermia plays a major role. NPS may also be inhibited during chronic ethanol consumption, but only if hypothermia and/or sufficiently high maternal blood ethanol levels are present. The role of ethanol-related hypothermia in human FAS remains to be elucidated.

Figure 1. Effect of Maternal Ethanol Intake on Fetal Organ Weight



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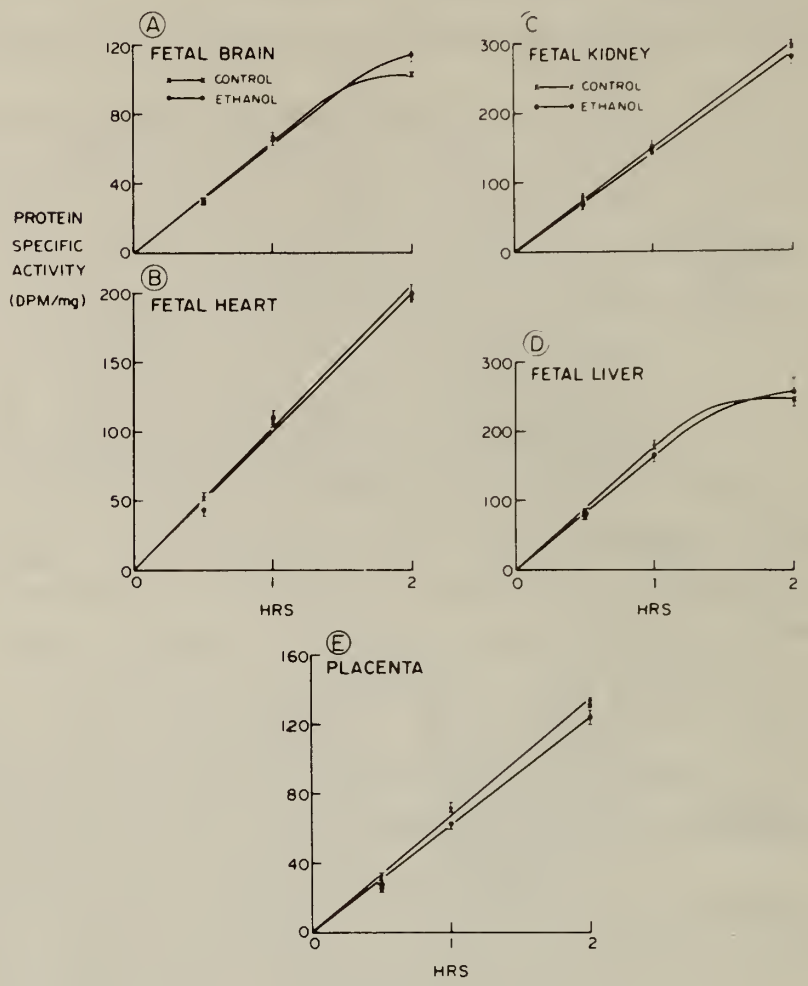
Note: The vertical axis represents the ratio of ethanol-exposed fetal organ weight to organ weights from control fetuses (A) and fetal organ weight: body weight ratios (B). Asterisks indicate a statistically significant ($p < 0.05$, students paired t test) difference between ethanol-exposed and pair-fed control values. Ethanol exposure times are indicated by gestation days 11 through 13 (n = 110 ethanol and 135 control fetuses), days 14 through 16 (n = 138 ethanol and 151 control fetuses), and chronic ethanol exposure (n = 135 ethanol and 179 control fetuses). Bars represent means ± SE.

Figure 2. Effect of Chronic Maternal Ethanol Intake on Fetal Brain, Visceral, and Placental Net Protein Synthesis



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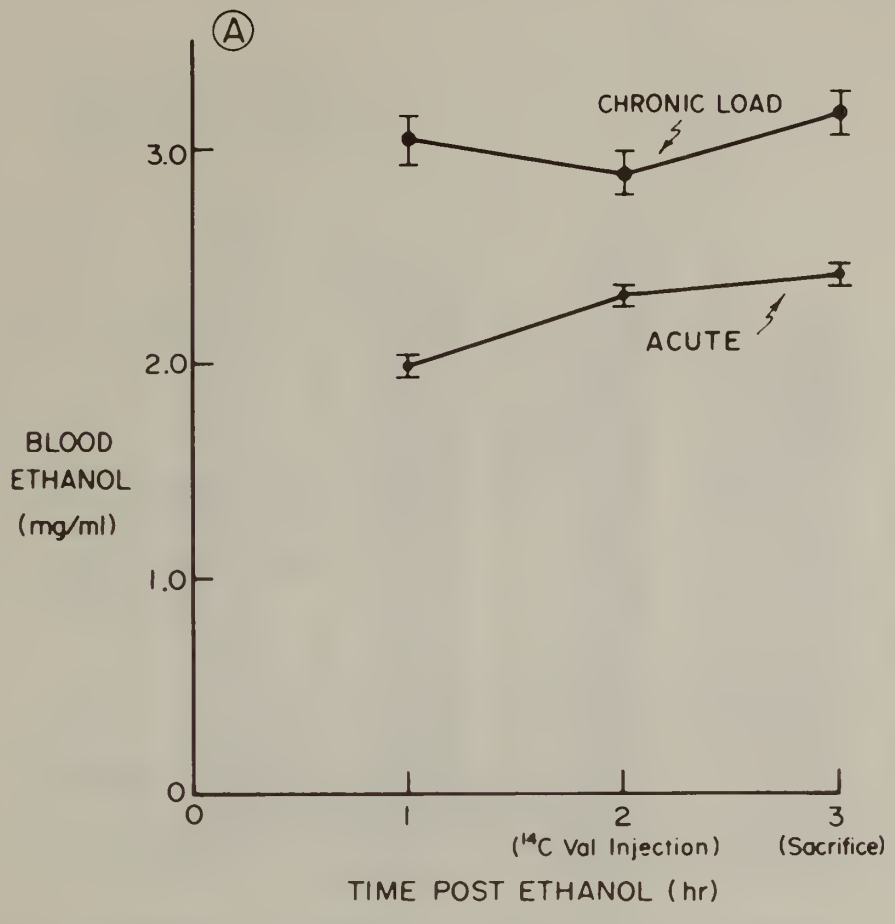
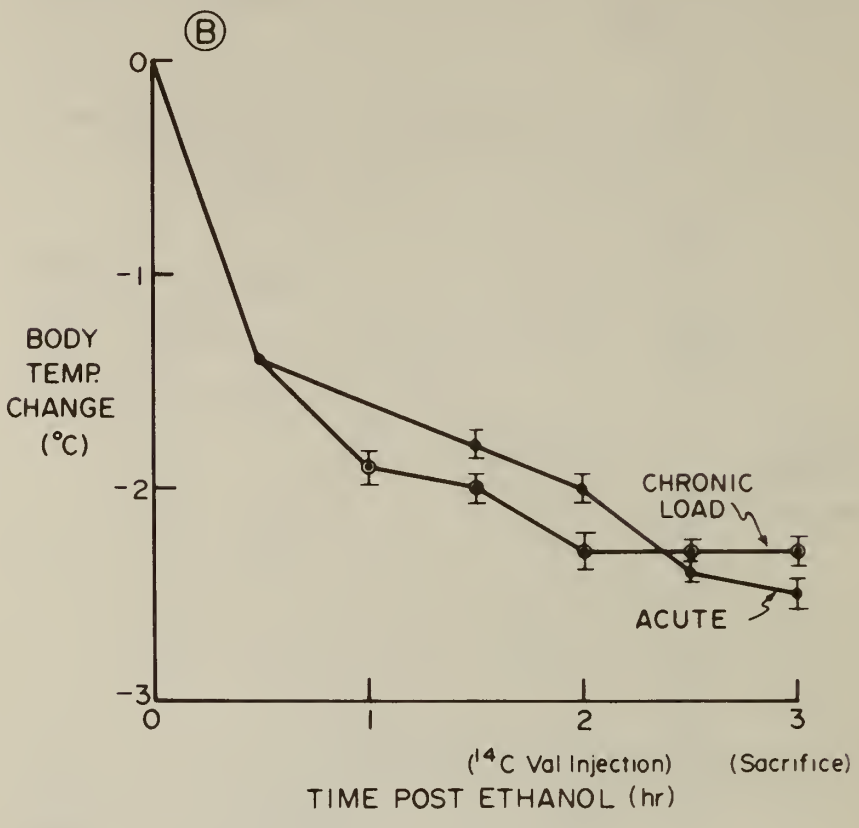
Note: The vertical axis represents the specific activity (dpm/mp protein) of extracted protein in fetuses chronically exposed in utero to ethanol (•——• ethanol) and in those from pair-fed control (X——X control) dams. Rats were sacrificed within 4 hours of the morning feeding when blood ethanol levels averaged 0.76 mg/ml and rectal temperatures were 38°C. The horizontal axis represents the time period (hours) between subcutaneal injection of (^{14}C) valine (500 mM; 5 μCi/mmol/100 g rat) and sacrifice. n = 113 ethanol-exposed fetuses and n = 98 control fetuses for the 0.5-hour time; n = 97 ethanol-exposed fetuses and n = 104 control fetuses for the 1-hour time; and n = 89 ethanol-exposed fetuses and n = 79 control fetuses for the 2-hour time. Vertical bars represent standard errors. The ethanol and control values did not differ statistically ($p > 0.05$).

Figure 3A. Effect of a Single Oral Dose of Ethanol on Blood Ethanol Levels



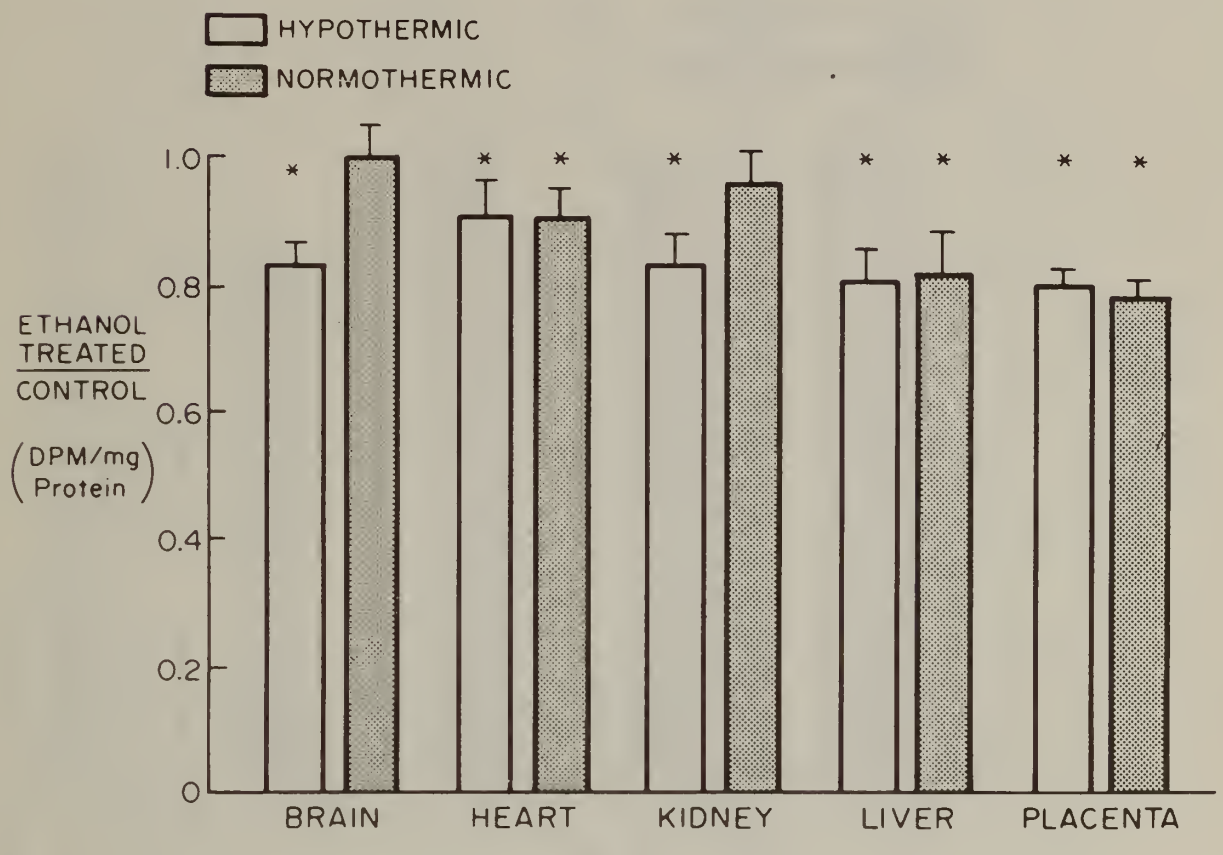
Note: The vertical axis represents blood ethanol levels (mg/ml), and the horizontal axis represents the time (hours) following a single peroral dose of ethanol (4 g/kg, 25 percent solution). The time that (^{14}C) valine was injected (2 hours) and the time of sacrifice (3 hours) following the administration of ethanol are indicated on the horizontal axis. Acute (•) represents the group of pregnant dams that were not allowed access to food the night prior to testing but received a single oral dose of dextrose (28.5 kcal/kg) in place of solid food. Chronic load refers to rats that had been maintained on an ethanol-containing diet prior to and during gestation (see "Methods") and were given a single oral dose of ethanol (4 g/kg) 3 hours prior to sacrifice. Vertical bars are standard errors of the mean. For chronic load, n = 7; for acute, n = 10.

Figure 3B. Effect of a Single Oral Dose of Ethanol on Body Temperature



Note: The vertical axis represents the change in rectal temperature (°C) from a norm of 38°C. The horizontal axis indicates the time (hours) following oral ethanol intake (4 g/kg, 25 percent solution). The time that ^{14}C-valine was injected (2 hours) and the time of sacrifice (3 hours) following the administration of ethanol are indicated on the horizontal axis. The groups of rats studied (acute and chronic load) are those described in figure 3A. Vertical bars are standard errors of the mean. For chronic load, n = 7; for acute, n = 10.

Figure 4. Effects of a Single Acute Dose of Ethanol and Concomitant Hypothermia on Net Protein Synthesis in Rats Chronically Exposed to Ethanol



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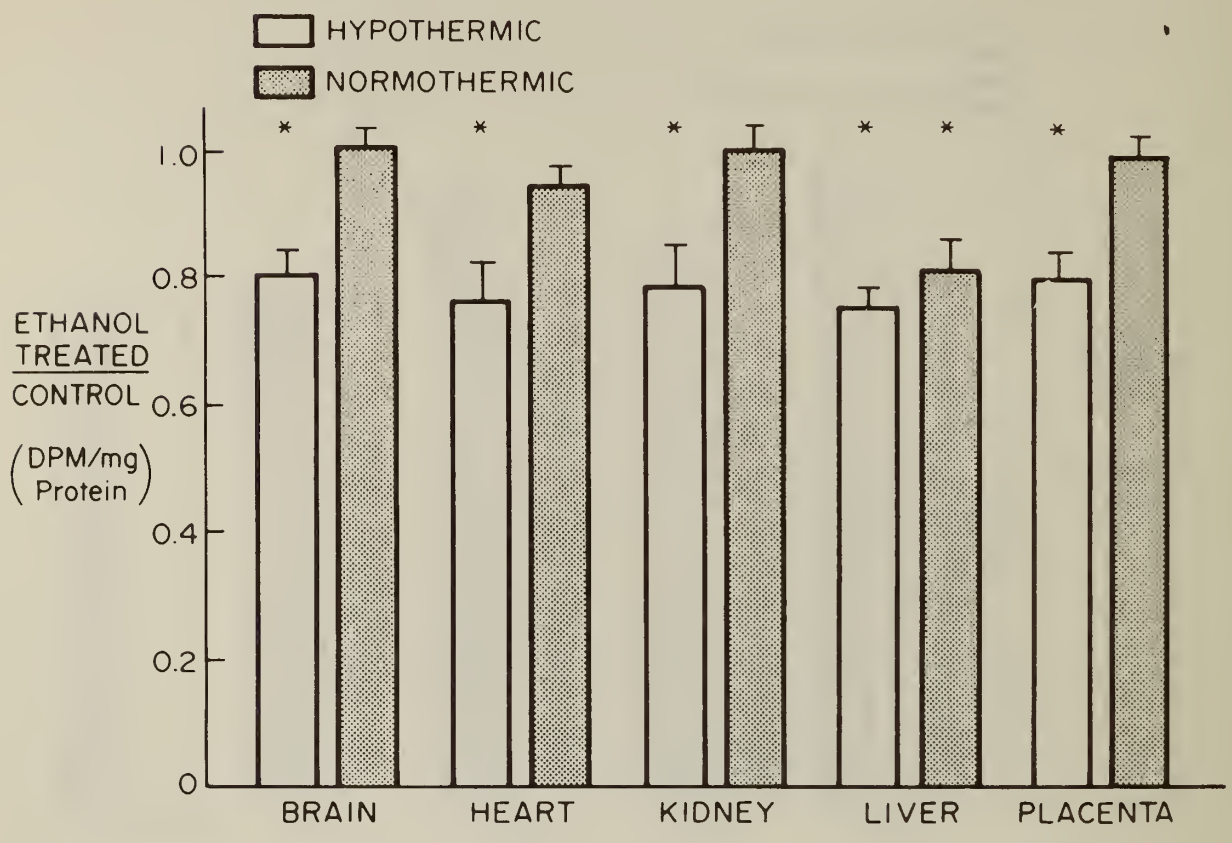
Note: The vertical axis represents the net protein synthesis expressed as a ratio of ethanol-exposed to pair-fed control values. Vertical bars indicate means ± SE. The horizontal axis indicates the organs studied and the treatment involved. The open bars refer to rats chronically exposed to ethanol that received a single acute dose of ethanol (4 g/kg, 25 percent solution) 3 hours prior to sacrifice and whose body temperature was allowed to drop to an average of 35.7 °C. The stippled bars refer to rats treated in a manner identical to those in the first group except that maternal body temperature was maintained at 38°C during the 3-hour period between ethanol administration and sacrifice. Maternal blood ethanol levels averaged 3.0 mg/ml for the hypothermic group and 3.2 mg/ml for the normothermic group. $n = 7$ pairs of hypothermic dams (80 ethanol and 87 control fetuses) and $n = 8$ pairs of normothermic dams (105 ethanol and 118 control fetuses). Asterisks indicate a statistically significant difference ($p < 0.05$) between ethanol-exposed and pair-fed control values for each organ.

Figure 5. Effect of a Single Dose of Ethanol and Concomitant Hypothermia on Net Protein Synthesis in Rats With No Previous Exposure to Ethanol



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Note: The vertical axis represents the net protein synthesis expressed as a ratio of ethanol-exposed to pair-fed control values. Vertical bars indicate mean ± SE. The horizontal axis indicates the organs studied and the treatment involved. The open bars refer to rats previously unexposed to ethanol and fasted prior to receiving a single acute oral dose of ethanol (4 g/kg, 25 percent solution) 3 hours prior to sacrifice; body temperature was allowed to drop spontaneously to an average of 35.8°C. The stippled bars refer to rats treated in a manner identical to those in the first group except that body temperature was maintained at 38°C during the time between ethanol administration and sacrifice. Maternal blood ethanol levels averaged 2.5 mg/ml for the hypothermic group and 2.7 mg/ml for the normothermic group. N = 10 pairs of hypothermic dams (112 ethanol and 108 control fetuses) and n = 10 pairs of normothermic dams (110 ethanol and 115 control fetuses). Asterisks indicate a statistically significant difference ($p < 0.05$) between ethanol-exposed and pair-fed control values.

Table 1. Effects of Ethanol Exposure on Fetal Viability

Ethanol Exposure Time	Group	N	Total Implants	Resorptions per 100 Implants	Dead Fetuses per 100 Implants	Nonviable Fetuses per 100 Implants	Fetal Weight (g) ($\bar{X} \pm SE$)	Maternal Ethanol Level mg/ml blood) ($\bar{X} \pm$ SE) Day 11	Day 12	Day 13
Days 11–13	Ethanol	12	138	20.3[a]	—	20.3[a]	2.0 ± 0.1[a]	0.98 ± 0.18	2.7 ± 0.5	3.7 ± 0.7
	Control	12	139	2.9	—	2.9	2.5 ± 0.1			
								Day 14	Day 15	Day 16
Days 14–16	Ethanol	15	144	4.2[a]	—	4.2[a]	2.0 ± 0.1[a]	1.1 ± 0.3	3.7 ± 0.5	4.1 ± 0.5
	Control	15	151	0	—	0	2.5 ± 0.1			
Chronic	Ethanol	18	181	21[a]	2.8	23.8[a]	1.0 ± 0.1[a]		0.7 ± 2.0[b]	
(20 days of gestation)	Control	18	187	2.7	1.6	4.3	2.5 ± 0.1			

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[a] $p < 0.01$.

[b] Minimum blood ethanol level before feeding (0.7 mg/ml blood) to maximum late in the dark cycle (2.0 mg/ml blood).

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Sequence Organization and RNA Transcripts of Mammalian Mitochondrial DNA*

Krishna K. Tewari and Robert Meeker

Abstract

Using the various sequence-specific restriction endonucleases Hind III, Hae III, Hha I, Bam HI, Hpa II, and Msp I, we have characterized and compared the mtDNAs of rat liver and brain. These studies have shown that no detectable sequence heterogeneity exists in the mtDNAs of these two tissues with any of these enzymes. The use of the isoschizomeric enzyme pair Hap II/Msp I to analyze for internal methylcytosine patterns showed no difference in the base sequences of mtDNAs of liver and brain. In the particular case of Hind III digests of mtDNA, we observed the production of six fragments of molecular weights 3.96-10^6, 2.50-10^6, 1.57-10^6, 1.26-10^6, 0.58-10^6, and 0.1-10^6 daltons. These carefully characterized fragments have been used to clone the brain mtDNA in pBR322. Redigestion of the stable clones with Hind III produced fragments comigrating with the native mtDNA fragments. When analyzed by electron microscopy, the measured lengths of the clone DNAs indicated that complete Hind III fragments were inserted into pBR322. Finally, digestion of the individual clone DNAs with Hae III gave further confirmation of the six Hind III mtDNA fragments and no apparent rearrangement of the mtDNA sequences during the cloning and isolation processes.

Mitochondria and chloroplasts are unique organelles in eukaryotic cells because they contain their own genetic system, i.e., their own DNA, enzymes to replicate and transcribe the genes, and the full enzyme complements to translate the organelle DNA into RNAs and proteins. Even though these organelles have most of their proteins encoded by nuclear DNA (nDNA), a few proteins are

NOTE: Figures and tables appear at end of paper.

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coded by the organelle DNA. Most of the elements of the mitochondrial genetic system have been identified in the past few years, and the genes in the mitochondrial DNA (mtDNA) have been identified and localized. The most recent studies have been able to completely sequence both the human and the bovine mitochondrial genomes. With all the understanding of the molecular biology of the mitochondrial genetic system, it is still puzzling as to why a eukaryotic cell has gone so far as to duplicate the replication, transcription, and translation system in both nuclei and mitochondria. In this review, we would like to present the information about the sequence organization of the mtDNA, its replication and transcription, and the RNAs coded by mtDNA. The review will deal exclusively with the mammalian mtDNA.

The Structure of Mitochondrial DNA

Mitochondrial DNAs from a number of mammalian organisms have been found to occur in the form of covalently closed circular duplex molecules (Borst 1977). Their structural organization results in the restricted uptake of the intercalating dye ethidium bromide, which forms the basis for a convenient centrifuge method for isolating pure organelle DNA. When DNAase-treated mitochondria are lysed with sarkosyl and centrifuged in a CsC1-EtBr density gradient, and the tube illuminated with near ultraviolet light, mtDNA is found to band at two distinct densities, as shown in figure 1. The upper band contains a mixture of nicked mtDNA and linear molecules, and the lower band contains closed circular mtDNA. Figure 2 shows electron micrographs from different parts of the CsC1-EtBr gradients to illustrate the kind of molecules obtained in the mtDNA (Clayton and Vinograd 1967). Most of the DNA molecules are monomers with some oligomers that are present mostly as circular dimers and catenated dimers. The mtDNA from practically all mammalian organisms have been found to be about 5 μ circles, which corresponds to a molecular size of 10×10^6. The recent sequence analysis of the HeLa cell mtDNA has established its size of 16,569 base pairs.

The mtDNA is an interesting example of gene redundancy. There are about 100 to 500 mitochondria in each mammalian cell. Each mitochondrion may contain from 2 to 10 molecules of DNA; thus, each cell contains about 1,000 mtDNA molecules. The question

arises as to whether all mtDNA molecules are identical in nucleotide sequence. This problem has been studied by several techniques involving earlier studies of quantitative DNA-DNA renaturation coupled with electron microscopy, denaturation mapping, and, more recently, restriction endonuclease digestion, and ultimately by the base sequence analysis. All of these studies strongly point out that all mtDNA molecules in a single organism are identical and that mtDNA contains no major gene repetitions. Thus, the potential genetic information of each mtDNA molecule is equivalent to its molecular weight. There are no distinctive or unusual bases in mtDNA. However, mtDNA molecules have been found to contain ribonucleotides, which make them alkali labile. The functional importance of ribonucleotides is not understood.

Replication of Mitochondrial DNA

The replication of closed circular mtDNA has been extensively studied (Borst 1972). The replication of mtDNA proceeds by displacement synthesis in the first discrete stage of DNA replication by having a short progeny strand hydrogen bonded at a unique site to the light parental strand (figure 3, B). This results in the formation of a displacement loop (D-loop). The closed D-loop mtDNA formed from the parent molecule by displacement replication of a hydrogen bonded, unique 450-nucleotide segment of heavy strand accumulates and accounts for about one-fourth of the mtDNA in exponentially growing mouse L cells (Robberson et al. 1972). Unidirectional displacement replication of heavy strand apparently then continues in the presence of a nicking system with expansion of the D-loop (figure 3). After at least 60 percent expansion has occurred, the synthesis of light strand begins at about 0.6 genome length from the origin and proceeds counterclockwise (figure 3, D and E). The replication of the two daughter strands in a single molecule is thus highly asynchronous, with a large distance ($8x10^3$ nucleotides) between the site for the initiation of heavy strand synthesis and the first site for the initiation of light strand synthesis. The separation of the daughter molecules from the Exp-D replicative structures (figure 3, F and G) occurs before light strand synthesis is completed. The products are L daughters and large gapped β Gpc molecules. The latter are then finished to form daughter molecules.

Sequence analysis of the HeLa mtDNA has provided more insight into the region of mtDNA involved in initiation of replication. In HeLa cell mtDNA, major 7S DNA species found in the D-loop correspond to nucleotide 191 of the DNA sequence (Anderson et al. 1981). The size of the 7S DNA ($<$680 nucleotides) and mapping data indicate that the 7S DNA and presumably the D-loop do not extend into the tRNA gene. The 1,100 base pair region of the human mt-genome between the $tRNA^{pro}$ and $tRNA^{phe}$ genes contains no large open-reading frames in either strand. It appears unlikely that this part of the genome codes for a major protein species. In the region covered by D-loop, several blocks of nucleotide sequence homology exist between the human, bovine, and rat mitochondrial genomes. However, the DNA separating these blocks are variable in length and exhibit no significant homology. The region between the 5′ end of the 7S DNA and the $tRNA^{phe}$ gene seems to be one of the least conserved regions of the entire genome. Very little sequence homology between the human, rat, or bovine mt-genome can be detected in this region. The size of the region also varies, being 385 nucleotides in the human placenta, 291 nucleotides in rat liver, and 180 nucleotides in beef heart. This region may contain control signals for the transcription of the mt-genome.

Restriction Endonuclease Maps of Animal Mitochondrial DNAs

The availability of restriction endonucleases with different base sequence specificities has enabled us to dissect the mtDNA into smaller DNA fragments and has ultimately paved the way for the sequencing of the genome. Brown and Vinograd (1974) used EcoRI and Hind III, which recognized different hexanucleotide sequences in DNA, to produce a physical map of mtDNA from established cell lines of human, African green monkey, and mouse origin. Because these DNA contain D-loops at the origin of replication, it was possible to develop cleavage maps of the fragments with the sites of origin of replication as reference points. Since replication proceeds unidirectionally, fragments obtained from larger replicating forms disclose the direction of replication of the map and refine the site of origin of replication to one fork of the D-loop. Cleavage maps based on EcoRI and Hind III endonucleases are presented in figure 4. It is apparent from figure 4 that mtDNA from different mammalian

systems have different restriction sites. Similar restriction endonuclease analysis has now resulted in detailed maps of HeLa cell, rat liver, and mouse mtDNAs. Ojala and Attardi (1977) identified 21 fragments among the products of digestion of HeLa cell mtDNA with the restriction enzyme Hpa II. The sum of the molecular sizes of these fragments, estimated from their mobility relative to that of known markers, accounted for the total length of HeLa cell mtDNA. The 21 fragments were ordered in a physical map by two approaches: (a) sequential digestion with Hpa II of the fragments produced by EcoRI, Hind III, and Hpa I enzymes; and (b) fragment-primed DNA synthesis. The Hpa II map was alined with the maps constructed with the other three enzymes using the unique cutting site produced by Bam HI. The map thus obtained has resolved HeLa cell mtDNA into 27 recognizable segments in the molecular size range between 75 and 1,950 base pairs. The physical map alined with the known positions of the rRNA and 4S RNA genes on the two mtDNA strands by RNA-DNA hybridization experiments, utilizing 32 P-labeled 12 and 16S rRNA, is presented in figure 5.

Similar studies on rat liver mitochondrial DNA have produced restriction endonuclease cleavage maps and identified the location of D-loops and of the rRNA and the tRNA genes. Francisco and Simpson (1977) reported that there were two types of mtDNA in the population of rats that could be distinguished by restriction endonuclease EcoRI analysis. These two types have been termed as types A and B mtDNA. Francisco et al. (1979) further analyzed the cleavage maps of A and B rat mtDNA. A comparison of types A and B maps shows that no major rearrangement has occurred since the time of the evolutionary segregation of the two DNAs. Analysis of the DNA fragments using a number of restriction endonucleases showed that there was no evidence of microheterogeneity in either of the mtDNA types. They estimated that the two types of mtDNA had sequence divergence amounting to about 0.94 to 1.8 percent. Nine enzymes yielded fragments, all of which could be accurately sized, leading to a reasonably accurate estimate of 15.85 kilo base pairs (kbp) for the size of rat liver mtDNA of either type. In a test of whether cytoplasmic inheritance is operative in mammals, reciprocal cross-experiments with the two DNAs showed that only the female parent mtDNA was inherited, with no evidence of persistence of the male parental mtDNA in any of the tissues examined. A detailed restriction endonuclease map of rat liver mtDNA has been obtained by Feldman and Grosskopf (1979) using XbaI, Bgl II, Hae II, Bam HI, Hpa I, Hha I, Bcl I, Hind II, Hind III, EcoRI, Hpa

II, Hae III, and Sau 3A (figure 6). It was derived from complete and partial digestion with these enzymes, double digestions, and redigestions of defined fragments obtained with one enzyme with other restriction enzymes. Maps of the restriction endonuclease digests of mammalian mtDNAs are thus.quite different from each other. In figure 7, the restriction sites of Bam HI, Hind III, and EcoR I in rat, mouse, HeLa cell, and bovine mtDNA are given for comparison.

From the foregoing description, it is quite clear that the mtDNA of a single animal are homogeneous in base sequences and the mtDNAs from different organisms have undergone significant sequence diversion. There is yet another question. Are the mtDNA sequences identical in different tissues of the same animal? It was reported that mtDNA from liver and heart would only cross hybridize to 90 percent, but hybridization is a very crude method of assessing DNA sequence conservation. Coote et al. (1979) have recently analyzed the mtDNA sequences between ox brain cortex and ox liver. Using EcoRI, Hind III, and Bam HI, they found that there was close similarity between the two types of DNA, and they both contained 16,000 base pairs. In seeking a clearer demonstration of any differences between brain and liver mtDNA another endonuclease, Hpa II, was tried. The Hpa II pattern differed slightly from one animal to another, but in each case the brain and liver from a single animal were significantly different. These data showed that the sequence CCGG does not occur in identical positions in brain and liver mtDNA from the same animal. We have (Meeker et al., in preparation) extended these studies to rat liver and brain mtDNA and utilized the restriction endonucleases Hind III, Bam HI, HhaI, Hpa II/MspI, and Hae III to detect any sequence differences between the mtDNAs from different organs. Our data shown in table 1 clearly indicate that there is no difference in the sequence organization of the two mtDNAs that could be detected by the use of restriction endonucleases.

RNA Transcripts of Mitochondrial DNA

Transcription of mtDNA is unique in that both strands are completely transcribed from a promoter situated in the D-loop region. These primary transcripts are then processed to give the

12S and 16S rRNAs, tRNAs, and a number of presumptive mRNAs that are not capped but are polyadenylated.

Ribosomal RNA

The HeLa cell mtDNA can be separated into two strands—H (heavy) and L (light) (Borst 1972). By DNA-RNA hybridization studies, H strand has been shown to contain one gene each for the 16S and 12S rRNA species. Both 16S and 12S rRNA genes were found to contain adjacent $tRNA^{val}$ and $tRNA^{phe}$, respectively. Eperon et al. (1980) have been able to aline the DNA sequence with the sequences of the 5′ ends of the 16S and 12S rRNAs. The analysis showed that there were no noncoding bases between the $tRNA^{val}$ gene and the 16S rRNA gene, and between $tRNA^{phe}$ gene and the 12S rRNA gene. It has also been shown that the mouse 12S rRNA gene abuts the 3′ $tRNA^{val}$ gene. Similarly, in hampster the 16S rRNA gene is close to 3′ $tRNA^{leu}$ gene. The sequencing of HeLa cell mtDNA has shown that there are no noncoding bases between the 16S and 12S rRNA genes. Thus the 12S rRNA and 16S rRNA and the flanking tRNAs in a primary transcript could be released by a cleavage precisely at the 5′ and 3′ ends of the tRNAs without the need for further processing. The sequences of the rRNA genes have been interpreted to provide support for a model in which mt ribosomes did not have sequence-dependent signals for the recognition of the current initiation codons, but rather initiated at the first initiation codon in the mRNA (Anderson et al. 1981). This is consistent with the known 5′ end sequences of the mRNAs (Montoya et al. 1981).

Transfer RNA

HeLa cell mtDNA has been shown to contain 22 tRNA genes by hybridization experiments with aminoacyl tRNAs labeled in the aminoacid moiety, visual inspection by electron microscopy, and sequence analysis. Bovine mtDNA has also been found to contain an equivalent number of tRNA genes. These tRNAs are sufficient to read all codons by using a mechanism unique to mitochondrial systems. Cytoplasmic tRNA have not been shown to be imported to mitochondria.

Two species of mammalian mt-methionyl tRNAs have been identified, a tRNA $^{met}_{i}$ and a tRNA $^{met}_{m}$. Hybridization of these tRNAs to mtDNA showed partial additivity, indicating the existence of two separate genes. In the DNA sequences of HeLa cell mtDNA (Anderson et al. 1981), only one $tRNA^{met}$ gene has been identified, presumably coding for the initiator tRNA. The sequences of the mammalian mt tRNAs are unusual in that all, except tRNA $^{leu}_{UUR}$, lack some or all of the following features found in other tRNAs: (a) the universal sequence G-T-Ψ-C-R-A; (b) the constant seven-base length of the 'T Ψ C' loop, which in mammalian mt tRNAs varies between three and nine bases; and (c) the constant bases A_{14}, G_{15}, and G_{18} G_{19} and the connections with U_8 and U_{28} (yeast $tRNA^{phe}$ numbering systems [Anderson et al. 1981]). Thus, the mt-tRNAs are apparently stabilized by fewer tertiary interactions. Even though homologous cytoplasmic tRNA species from different animals are quite similar, there are substantial variations in specific tRNAs from human and bovine mitochondria.

Messenger RNA

The fractionation of HeLa cell mtRNA labeled with 32P-orthophosphate in the presence of camptothecin (to block all high-molecular-weight nuclear RNA synthesis) by high-resolution agarose-CH_3HgOH electrophoresis is shown in figure 8 (Attardi et al. 1979). At least 18 bands of varying intensity can be recognized in the autoradiogram of the oligo (dT)-bound RNA fraction (figure 8, a) and 14 bands in that of the nonbound RNA fraction (figure 8, b). Most prominent among the latter are the two rRNA species, 16S (VIII) and 12S RNA (XIII); the 4S RNA, under the conditions of electrophoresis used here, has run out of the gel. The oligo (dT)-bound RNA has been shown to contain poly(A) stretches corresponding to about 55 nucleotides by digesting it with pancreatic and T_1 ribonuclease followed by polyacrylamide gel electrophoresis. C and d in figure 8 show the autoradiograms of the oligo (dT)-bound and unbound RNA from polysomes separated from the mitochondrial Triton-X 100 lysate by sucrose gradient centrifugation. In general, the same components can be seen, with the exception of components 1 to 4 in poly(A) fraction, which are probably not mitochondrial. Figure 8, e, shows the electrophoretic pattern obtained for the poly(A)-containing RNA fraction extracted from

the micrococcal-nuclease-treated mitochondrial fraction of HeLa cell labeled with 32P-orthrophosphate in the absence of inhibitors. These RNA species have been shown to be truly of mitochondrial origin by their complete sensitivity to ethidium bromide and, more significantly, their sequence homology to mtDNA. The molecular weights of the poly(A)-containing RNA species, as estimated by comparison with the electrophoretic mobility of known standards, are shown in table 2, along with the sequence complementarity of these RNA species to separated mtDNA strands. With the exception of the largest species, 1, 2, and 3, and the smallest component 18 (7S RNA), all of the discrete poly(A)-containing RNA species are complementary to the H strand. Five mt poly(A) RNA species have been identified to code for known proteins of the mitochondria. The subunits of cytochrome oxidase I, II, and III are coded by mRNA numbered 9, 16, and 15, respectively (figure 9). RNA species 11 codes for the subunit of cytochrome b, and the RNA number 14 codes for the sixth subunit of ATPase. The sequence analysis of HeLa cell mtDNA has shown 13 significant reading frames, 8 of which are for unidentified proteins.

In regions representing identified genes, the respective human and bovine sequences are highly homologous. As shown in table 3, there is 80 percent amino acid conservation, which is 5 percent higher than the nucleotide conservation, reflecting the degeneracy of the genetic code. Over half of the codons for the conserved amino acids are different in the third position or, in the case of leucine codons UUR and CUR, in the first and third positions.

The sequence analysis of HeLa cell and bovine mtDNA has identified eight unidentified reading frames. On average, these show 70 percent nucleotide and amino acid conservation; half of the conserved amino acids have changed codons. These reading frames are certainly coding for mitochondrial proteins because the mapping and sequence analysis of the polyadenylated presumptive mRNAs (Montoya et al. 1981) corresponds exactly to the reading frames of URF 1, URF 2, CO I, CO II, URF A6L, and ATPase 6, CO III, URF III, URF4L, and URF 5 and the antisence of URF 6 and cytochrome b. In addition, large L-strand transcripts cover the H-strand-coded URF6, and the number and sizes of these reading frames are in fairly good agreement with the band pattern obtained by gel electrophoresis of HeLa cell mt-protein produced in the presence of emetine.

Unique Organization of Mitochondrial DNA

Certain features of the mt-genome are unique to the mitochondrial system. Analysis of an almost complete mammalian mtDNA sequence has identified 23 possible tRNA genes that are sufficient to translate all the codons of the mitochondrial genetic code (figure 9). This number is much smaller than the minimum of 32 required by the wobble hypothesis. For each of the eight genetic code boxes with four codons for one amino acid, it has been found that there is a single specific tRNA gene with T in the first (wobble) position of the anticodon. Therefore, it has been suggested that these tRNAs with U in the wobble position can recognize all four codons in these genetic code boxes either by a two-out-of-three base interaction or by U-N wobble.

The initiation codons are also different in the mt-genome. The sequence analysis of mtDNA (Anderson et al. 1981) has shown that all the identified genes (except ATPase 6) and some of the URFs have an AUG initiation codon within the first six bases in their mRNA start; others do not. The first AUG in bovine URF2 is 320 codons downstream from the RNA start in a reading frame of 347 codons. Similarly, in human and bovine URF3 the first AUG is, respectively, 89 and 87 codons downstream in a reading frame of 115 codons. All reading frames that do not have an AUG near the 5′ end of the RNAs have an AUA in an equivalent position. Thus, if these reading frames are translated completely, as their homology suggests they are, AUA must function as an intiation codon as well as AUG. AUA has already been shown to code for internal methionine as well as AUG. In the case of human URF2 there is an AUU in the same position as the AUA in bovine URF2. Thus, to translate completely this reading frame, AUU, like AUA, can also be an initiation codon in mt-genes. In mitochondria, over half of the presumptive mRNAs do not have UAA or UAG termination codons at the end of the reading frame. In three cases, human COI and URF6 and bovine cytochrome b, termination probably occurs at AGA and AGG codons. These have been predicted to be termination codons and not arginine codons as in the universal genetic code. This finding is based on the observation that only CGN arginine codons are used in all the reading frames, and AGA and AGG are only found at the end of reading frames at the junction with the next gene or where the reading frame continued into the next gene.

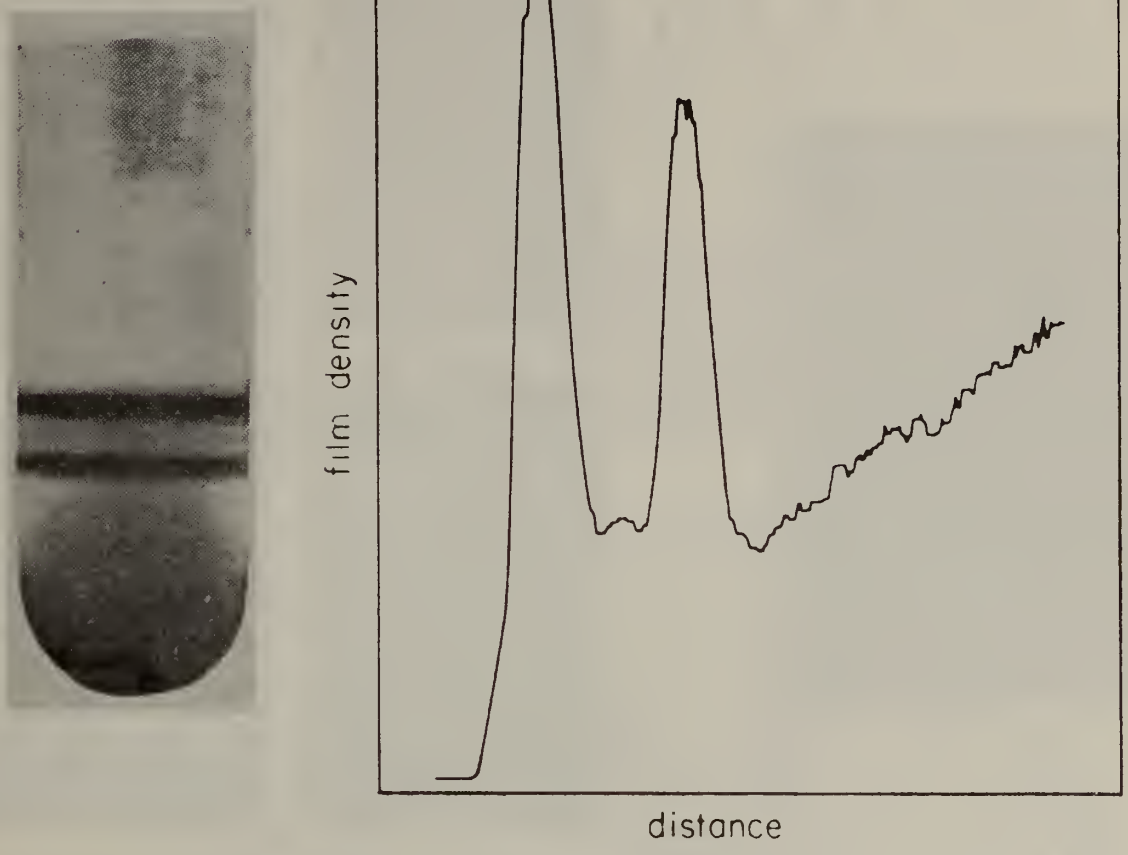
The mtDNA has another interesting feature. The genes in the mtDNA are closely spaced and tRNA genes punctuate the reading of genetic information in human mtDNA (Ojala et al. 1980). A detailed transcription map of HeLa cell mtDNA has been constructed by using the S_1 protection technique to localize precisely the sequence coding for the rRNA and poly(A)-containing species on the physical map of the DNA. This transcription map has been correlated with the position of tRNA genes derived from the mtDNA sequence. It has been shown that, with the exception of the D-loop and another small segment near the origin of replication, the mtDNA sequences are completely saturated by the rRNAs, poly(A)-containing RNAs and tRNAs coded for by the two strands. The data have been found to be consistent with a model of transcription of the H-strand in the form of a single molecule, which is processed into mature RNA species by precise endonucleolytic cleavages, occurring in almost all cases immediately before and after a tRNA sequence. This proposal has been further confirmed by sequencing the 3′ end of most of the putative mRNAs (Ojala et al. 1981). In all cases, the 3′ end nucleotide of the individual mRNA coding sequence has been found to be immediately contiguous to a tRNA gene or another mRNA coding sequence. To explain the lack of termination codons in most of the reading frames, it has been suggested that UAA termination codons are created by posttranscription modification. If the tRNAs are precisely cleaved out of a primary transcript by an RNase(P)-type enzyme(s), mRNAs with no termination codons would be left with either a U or UA at 3′ ends in phase with the reading frame. A polyadenylation of these RNAs would create a UAA codon.

Summary

From the foregoing description, it is quite clear that the mammalian mitochondrial genome has a unique organization and differs from other known genetic systems. The mammalian mt genetic code is unique, with its own initiation and termination codons. The arrangement of genes in mammalian mtDNA is quite distinctive and is not found in any other biological system. We have understood a large amount of the structural and functional organization of mtDNA. However, the biological necessity of

duplicating all the genetic machinery in mitochondria is yet to be explored.

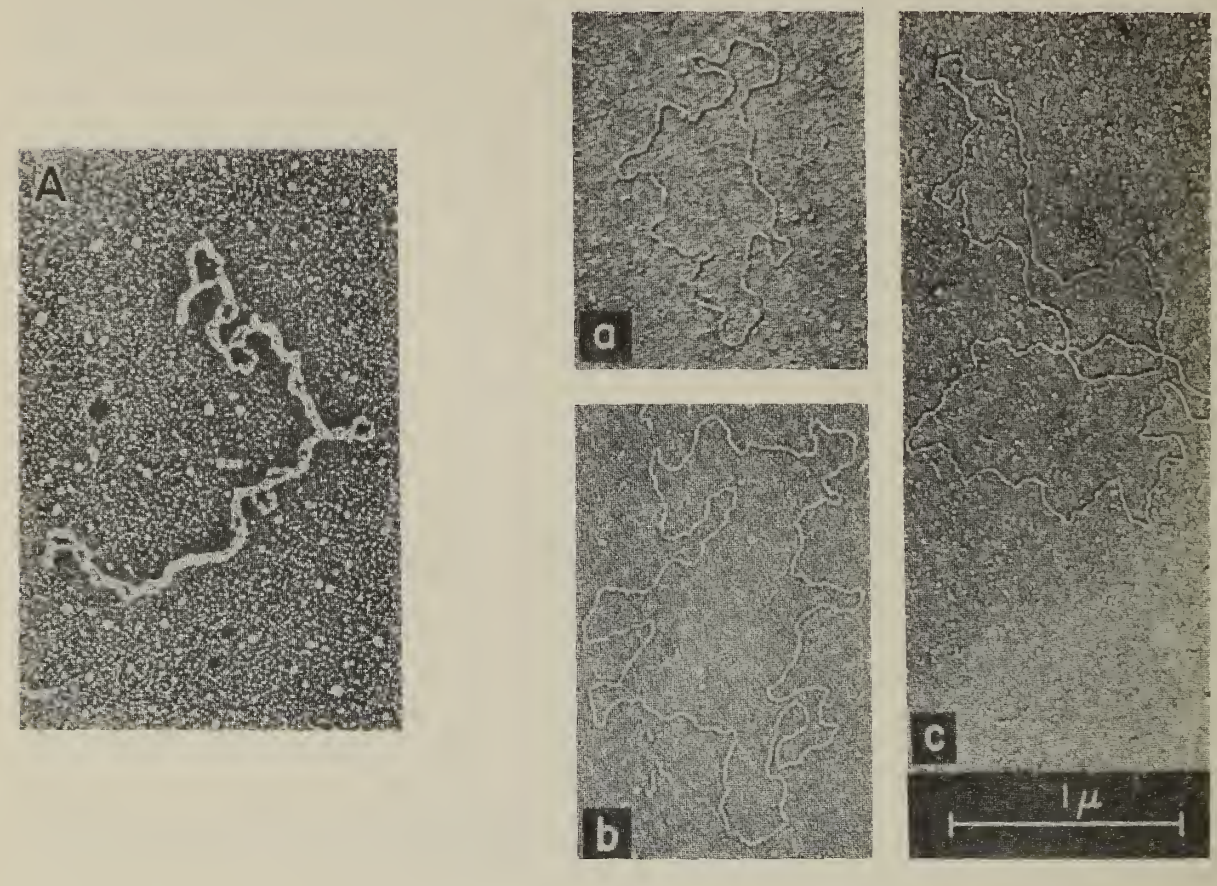
Figure 1. Flourescence Photograph of a Cesium Chloride Ethidium Bromide Density Gradient



SOURCE: Clayton and Vinograd 1967. Reprinted by permission from *Nature,* Vol. 216, p. 652. Copyright © Macmillan Journals Limited.

Note: On the right is a microdensitometer tracing showing band positions. The left-hand side of the tracing corresponds to the upper part of the tube.

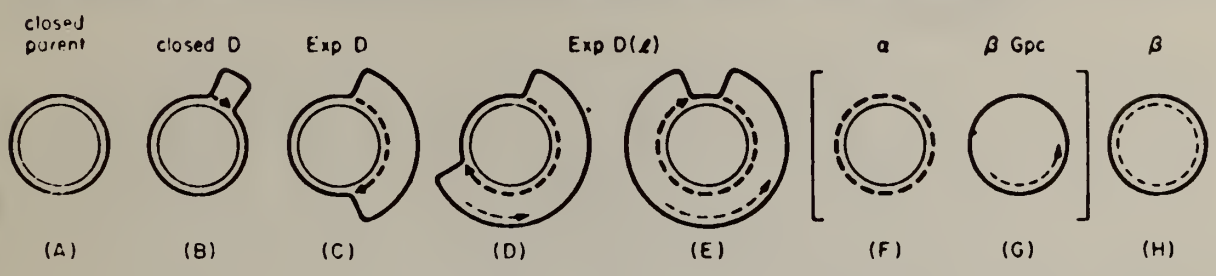
Figure 2. Electron Micrographs of Mitochrondrial DNA From CsC1-EtBr Density Gradient of Figure 1



SOURCE: Clayton and Vinograd 1967. Reprinted by permission from *Nature,* Vol. 216, p. 652. Copyright © Macmillan Journals Limited.

Note: A, supercoiled DNA from lower band; a, single length mtDNA from the upper band; b, circular dimer form; c, catenated dimer.

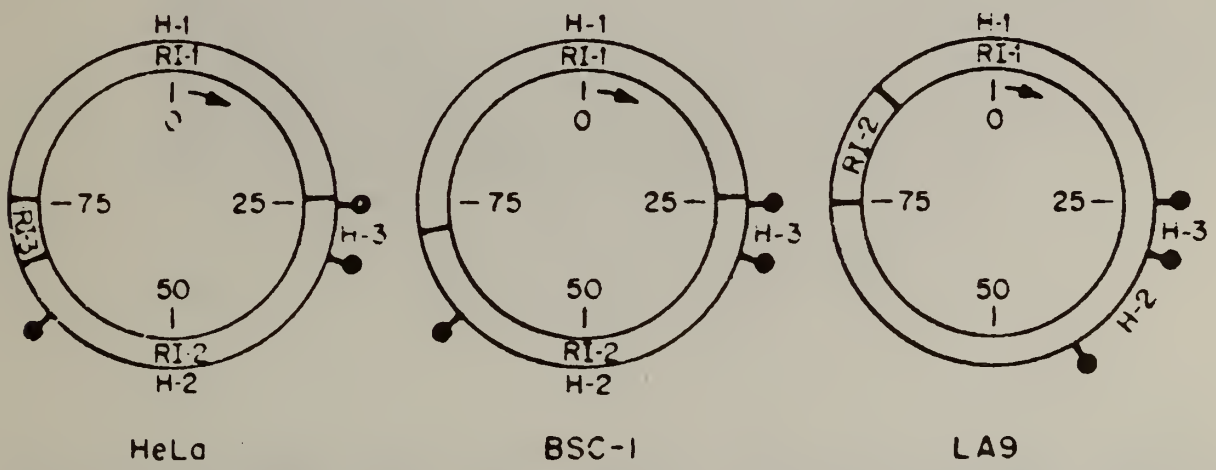
Figure 3. Replication of L Cell mtDNA



SOURCE: Robberson et al. 1972.

Note: Parental strands are solid lines; progeny strands are dashed lines; heavy strands are thick lines; and light strands are thin lines. Nicks and small gaps are not indicated. Daughter molecules (F and G) are represented as having formed after separation occurred in a completely expanded D-loop molecule. Exp-D(1), an expanded D-loop molecule in which light strand synthesis has occurred; Gpc, gapped circular molecule.

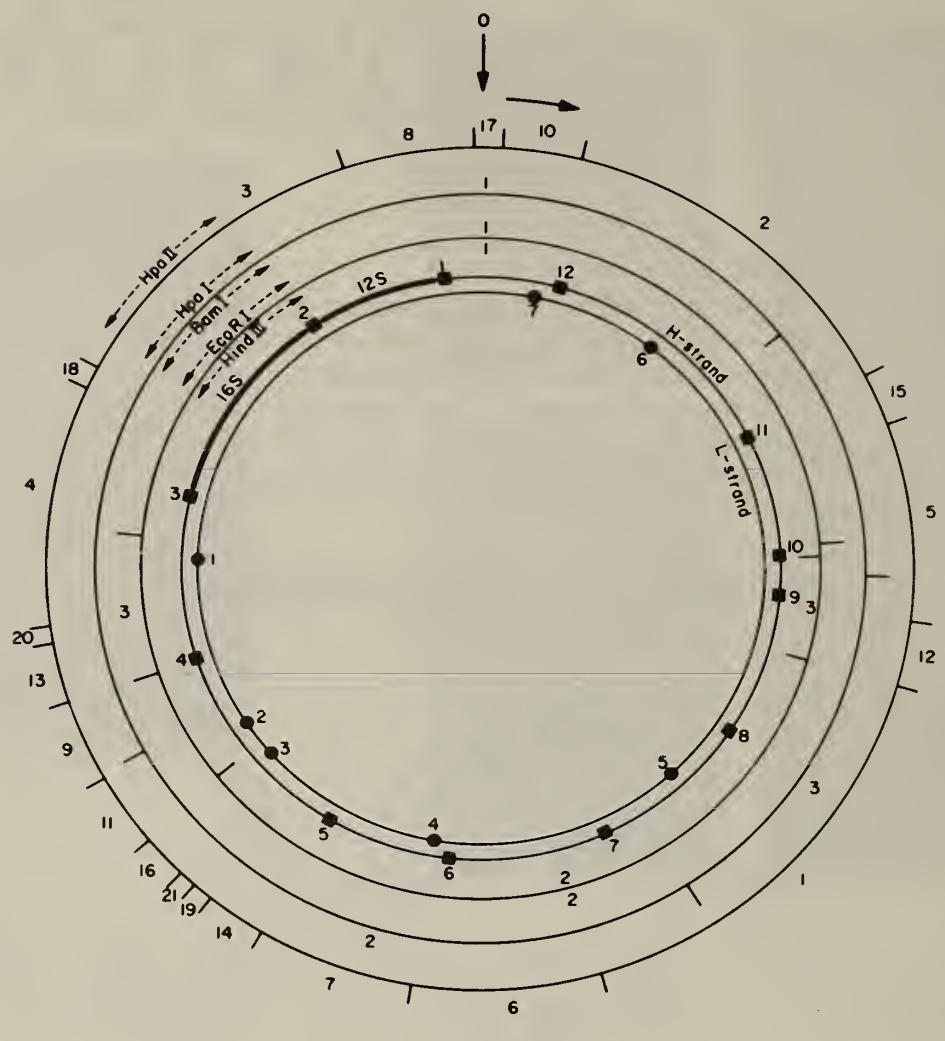
Figure 4. Cleavage Maps of Three mtDNAs



SOURCE: Brown and Vinograd 1974.

Note: O represents the origin of replication and the arrow represents the direction of replication. EcoRI restriction sites are shown in concentric circles. The Hind III sites are indicated by the symbol •.

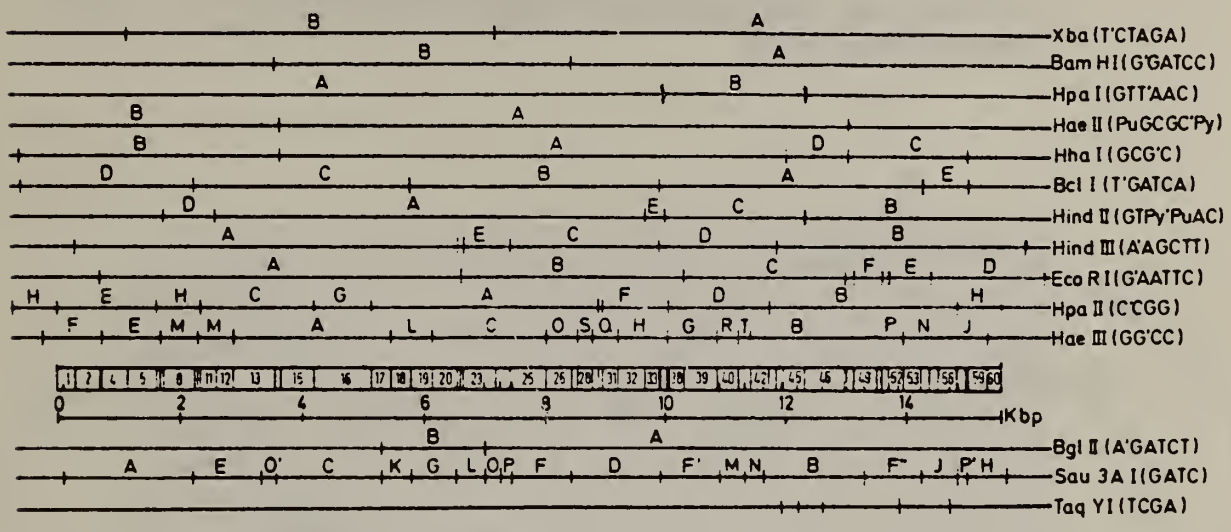
Figure 5. Restriction Maps of HeLa Cell mtDNA and Map of the Positions of the rRNA and 4SRNA Genes



SOURCE: Ojala and Attardi, *Plasmid,* 1:78, 1977. Copyright 1977 by Academic Press.

Note: The vertical arrow (marked O) and the rightward arrow indicate the origin and direction of the H-strand mtDNA synthesis.

Figure 6. Linearized Presentation of the Restriction Endonuclease Cleavage Map of Rat Liver mtDNA

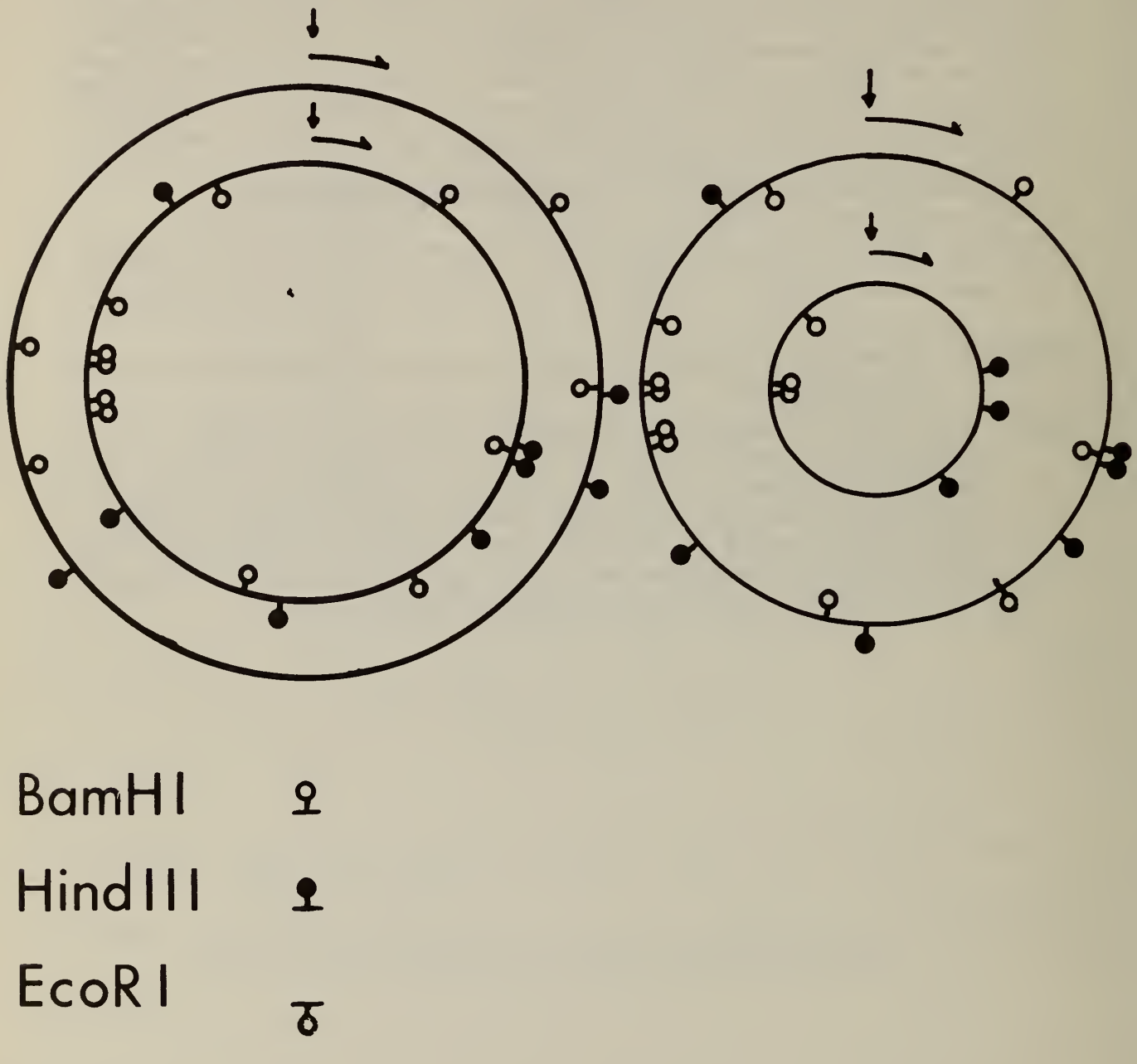


SOURCE: Feldman and Grosskopf 1979. Copyright 1979 by Springer-Verlag New York, Inc.

Note: The series of fragments obtained by digestion of mtDNA with a particular restriction endonuclease are designated by capitals. The subfragments produced in the upper part of the map are designated by arabic numerals, taking the restriction site between the Hpa II fragments H and E as reference point. In the lower part of the map, those fragments and sites are listed that were derived from rediges-tions of isolated fragments with further enzymes.

400-552 O - 83 - 5 QL 3

Figure 7. Left: Restriction Endonuclease Map of Rat (inner circle) mtDNA and Human (outer circle) mtDNA. Right: Restriction Endonuclease Map of Mouse (inner circle) mtDNA and Rat (outer circle) mtDNA



Note: Sites are indicated in figure as (↓), origin of replication; (→), direction of heavy strand synthesis.

Figure 8. **Autoradiograms After Electrophoresis Through Agarose CH_3HgOH Slab Gels, of Mitochondrial Poly(A)-Containing and Non-Poly(A)-Containing RNAs**

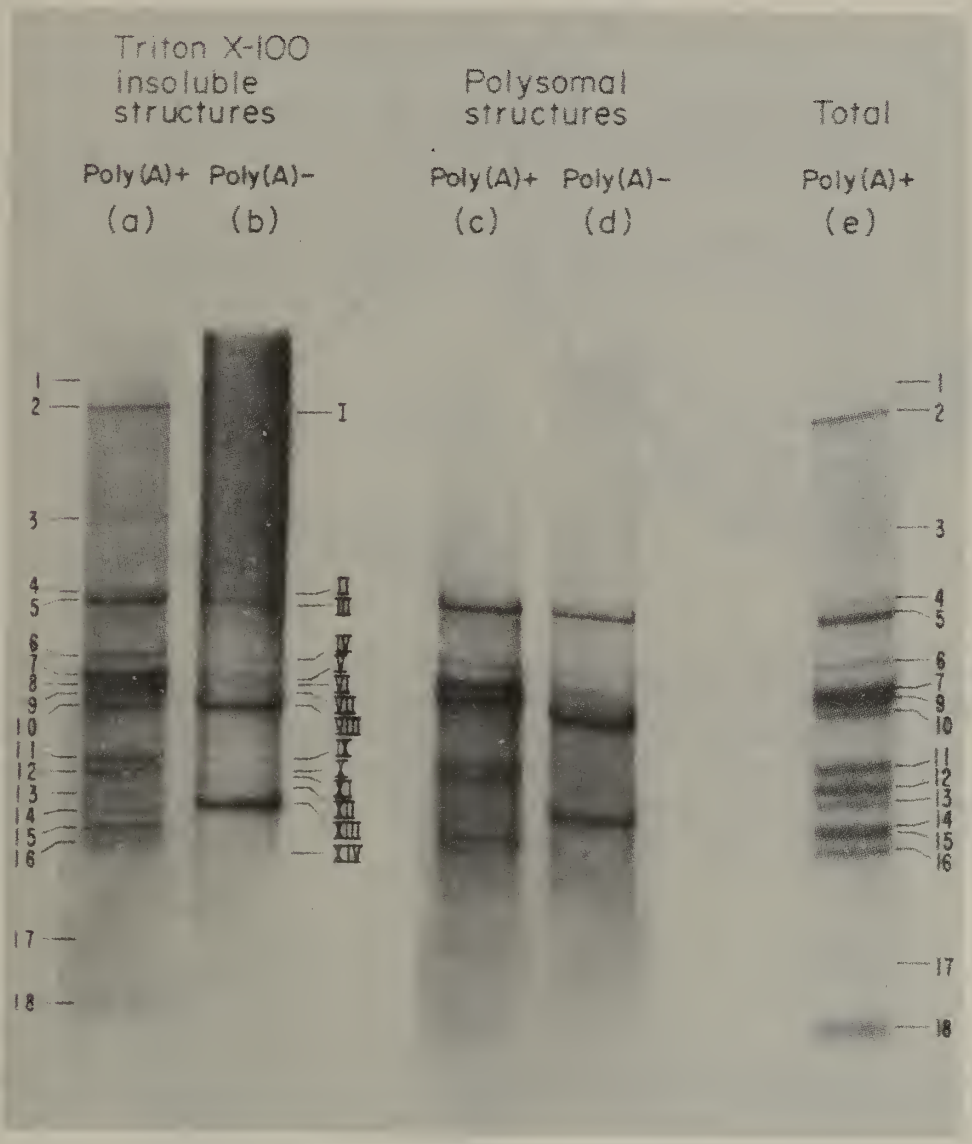
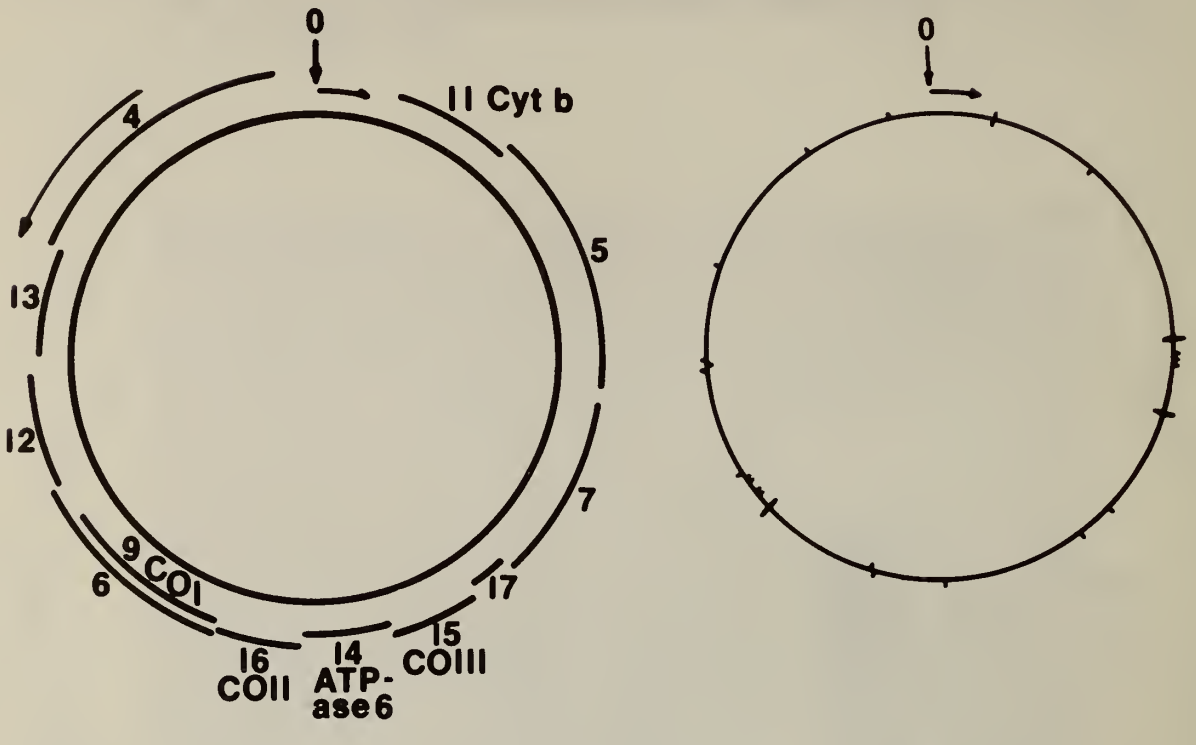


Figure 9. Physical Map of the Heavy Strand Transcripts of HeLa Cell mtDNA



Note: Known function transcripts are COI, cytochrome oxidase I; COII, cytochrome oxidase II; COIII, cytochrome oxidase III; and cytochrome b. Right: Physical map of transfer (t) RNA genes of HeLa cell mtDNA. H strand tRNAs (⊥), L Strand tRNAs (⊤), and Hind III restriction endonuclease sites (+). O shows origin of replication; → shows direction of H strand synthesis.

Table 1. Rat mtDNA Restriction Fragments: Molecular Sizes of Various Restriction Fragments Obtained With Liver and Brain mtDNA, Using a Number of Endonucleases

Restriction Enzyme	Hind III (kbp)[a]		Bam HI (kbp)		HhaI (kbp)		Hpa II/MspI (kbp)		HaeIII (kbp)		HaeIII/Hind III (kbp)	
Organ	Liver	Brain	Liver	Brain	Liver	Brain	Liver	Brain	Liver	Brain	Liver	
	6.19	6.19	11.56	11.56	6.56	6.56	4.06	4.06	2.64	2.64	2.58	0.30
	3.91	3.91	5.00	5.00	4.37	4.37	3.44	3.44	2.16	2.16	1.69	0.27
	2.39	2.39			2.08	2.08	2.01	2.01	1.83	1.83	1.33	0.21
	1.97	1.97			1.84	1.84	1.80	1.80	1.36	1.36	0.91	0.20
	0.83	0.83			1.06	1.06	1.72	1.72	0.92	0.92	0.81	0.17
	0.16	0.16					1.13	1.13	0.80	0.80	0.78	
							0.87	0.87	0.77	0.77	0.77	
							0.63	0.63	+0.67	+0.67	0.67	
	15.45	15.45	16.56	16.56	15.91	15.91	15.66	15.66	0.61	0.61	0.66	
									0.58	0.58	0.59	
									0.55	0.55	0.58	
									0.53	0.53	0.55	
									0.39	0.39	0.52	
									+0.37	+0.37	0.51	
									0.34	0.34	0.49	
									0.32	0.32	0.41	
									+0.22	+0.22	0.40	
									0.20	0.20	+0.37	
									+0.17	+0.17	+0.33	
									16.86	16.86		16.80

[a] kbp = kilo base pairs.

Table 2. Molecular Weight and Strand Specificity of Mitochondrial DNA Coded Poly(A)-Containing RNAs

Poly(A)-Containing RNA Component	Molecular Weight $\times 10^{-5}$	Coding Strand
1	34.0	L
2	28.0	L
3	14.0	L
4	9.0	H
5	8.6	H
6	6.7	H
7	6.2	H
8	6.0	H
9	5.8	H
10	5.4	H
11	4.2	H
12	4.0	H
13	3.6	H
14	3.3	H
15	3.2	H
16	2.9	H
17	1.4	H
18	0.93	L

Table 3. Characteristics of Human mt Genes and Comparison With Bovine mt Genes

Gene	Protein Length	Molecular Weight	Percent Amino Acid Conservation	Initiation and Termination Codons			
				Human		Bovine	
URF1	318	35,600	75.79	ATA		ATG	
URF2	347	38,900	62.82	ATT		ATA	
CO1	513	57,000	91.23	ATG	AGA	ATG	TAA
CO11	227	25,500	72.69	ATG	TAG	ATG	TAA
URFA6L	68	7,900	51.52	ATG	TAG?	ATG	TAA?
ATTase 6	226	24,800	77.88	ATG		ATG	
CO111	261	30,000	86.97	ATG		ATG	
URF3	115	13,200	73.91	ATA		ATA	
URF4L	98	10,700	73.47	ATG	TAA?	ATG	TAA
URF4	459	51,400	74.07	ATG		ATG	TAA
URF5	603	66,600	69.49	ATA	TAA	ATA	AGA
URF6	174	18,600	62.64	ATG	AGG	ATG	
Cytochrome b	380	42,700	78.10	ATG		ATG	

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GROUP 2: ETHANOL AND PROTEIN SYNTHESIS IN EUKARYOTES

Eukaryotic Protein Synthesis: An Overview*

Kivie Moldave

Abstract

The translation of natural mRNA in eukaryotic systems requires a series of interactions involving various enzymes, ribosomal subunits, aminoacyl-tRNAs, translational protein factors, nucleotides, etc., that carry out specific reactions leading to peptide chain initiation, elongation, and termination. One of these aminoacyl-tRNAs, Met-tRNA$_f$, is used in conjunction with ribosomal subunits specifically for the initiation of translation. The intermediary reactions in initiation in eukaryotic cells involve the formation of a ternary complex composed of Met-tRNA$_f$, eIF-2, and GTP, then binding of the ternary complex to 40S subunits, the subsequent addition of mRNA, and finally, the joining of 60S subunits. Each of these interactions requires one or more initiation factors, and the product formed is an 80S initiation complex containing mRNA and the initiator tRNA, Met-tRNA$_f$. Chain elongation requires GTP, elongation factors EF-1 and EF-2, and all the aminoacyl-tRNAs besides Met-tRNA$_f$. Elongation factor EF-1 catalyzes the binding translocation factor; both require GTP, which is hydrolyzed in the process. Aminoacyl-tRNAs bound to the A site of the ribosome react with peptidyl-tRNA at the P site, in a reaction (peptidyltransferase) catalyzed by the 60S ribosomal subunit, to form a new peptidyl-tRNA one amino acid longer at the A site. The newly formed peptidyl-tRNA is translocated to the P site, making the A site available for a new aminoacyl-tRNA. This series of reactions is repeated until all of the internal codons in mRNA are translated. Chain termination does not require any aminoacyl-tRNAs; recognition of the termination codon is carried out by a protein factor that interacts with ribosomes and leads to the release of the completed polypeptide chain. Preparations capable of carrying out translation of mRNA and individual intermediary reactions are available. The re-

NOTE: Figures and table appear at end of paper.

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sponse to alcohol varies, depending on the system. For example, translation of mRNA in preparations from a cultured mammalian cell is inhibited in relatively low levels of alcohol; however, the peptidyltransferase reaction in a highly purified, isolated system is markedly stimulated by relatively high alcohol concentrations.

Introduction

The past decade has seen rapid and remarkable progress toward an understanding of the process of protein synthesis in mammalian cells. Several excellent reviews have appeared recently that describe these contributions in detail (Jackson 1975; Lodish 1976; Revel and Groner 1978; Safer and Anderson 1978; Weissbach and Ochoa 1976). The reader is referred to these reviews for a more detailed treatment of the pathways, reactions, and components that are involved, as well as references to much of the original research. This preparation is intended to provide only a general analysis of the steps involved in this complex series of reactions.

In protein synthesis, the information encoded in the nucleotide sequence in mRNA is translated into amino acids that are polymerized into a polypeptide chain. The first step is that amino acids are activated and attached to cognate tRNAs, in reactions catalyzed by specific aminoacyl-tRNA synthetases (figure 1); the carboxyl groups of free amino acids are activated in the presence of ATP, via the formation of aminoacyl-adenylate intermediates, and the aminoacyl moieties are then transferred to the 2′(3′)-hydroxyl group of the 3′-terminal adenosine of specific acceptor tRNAs. The amino acid activation reaction and aminoacylation of tRNAs are described elsewhere in this volume. There are two important consequences of the formation of aminoacyl-tRNAs: the ester bonds by which the amino acids are attached to tRNA are "high energy" bonds, which are used for the subsequent formation of peptide bonds; and the amino acids are attached to specific tRNAs, which participate as "adaptor" molecules responsible for the recognition of appropriate nucleotide triplets in mRNA, by base pairing with complementary triplet bases, placing the amino acids in the correct position in the polypeptide sequence.

The translation of mRNA involves three distinct phases—initiation, elongation, and termination—each requiring individual components and reactions as depicted schematically in figure 1. One of the aminoacyl-tRNAs, the initiator methionyl-$tRNA_f$, reacts with ribosomal subunits, GTP, and initiation factors to decode the

initiation codon ApUpGp in mRNA. The product of these series of reactions is an 80S initiation complex containing a ribosome, mRNA, and Met-tRNA$_f$, in which the first meaningful codon in mRNA has been translated into an amino acid; further, the reactions result in the appropriate phasing of the protein synthesizing apparatus on mRNA so that all of the subsequent codons are read in the correct triplet sequence. The 80S initiation complex and all the other aminoacyl-tRNAs are used to translate the internal codons in mRNA, subsequent to the one specifying initiation. In the presence of elongation factors (distinct from initiation factors) and GTP, the aminoacyl moieties of aminoacyl-tRNAs are linked one at a time to the methionine in the 80S initiation complex; this involves a cycling series of reactions that is repeated many times as the ribosome moves on mRNA, a triplet at a time from the 5′ to the 3′ direction, until all of the meaningful codons have been translated. As chain elongation proceeds, and the ribosome has moved some distance from the initiation codon, another series of initiation reactions can occur, and a new 80S initiation complex at that site is formed from ribosomal subunits and a new Met-tRNA$_f$, as a consequence of their interaction with catalytically active initiation factors. Subsequently, a number of ribosomes can thus associate with a single mRNA, simultaneously producing several copies of the same polypeptide. The polyribosomal complexes (polysomes) are also described elsewhere in this volume. When all of the meaningful codons in a given mRNA have been translated, the ribosome comes to a termination codon and, in the presence of release factor(s) and GTP, the completed polypeptide chain is released.

Polypeptide Chain Initiation

Ribosomes (80S ribonucleoprotein particles) are dissociated to 40S and 60S ribosomal subunits by initiation factor eIF-3, or they dissociate spontaneously to subunits and are prevented from reassociating by eIF-3 (Thompson et al. 1977), as shown in the upper left portion of figure 2. The exact role of eIF-3, either as a dissociation or an antiassociation factor, is debatable; however, eIF-3 on 40S subunits prevents the joining reaction with 60S subunits, allowing all of the following intermediary reactions in chain initiation, which are specific for the small (40S) ribosomal subunit, to take place. The 40S subunit interacts with a ternary complex

composed of initiation factor eIF-2, GTP, and the initiator Met-tRNA$_f$. This reaction places the Met-tRNA$_f$ at a site on the 40S subunit that will subsequently form the "P" or "donor" site on the 80S ribosome (upper right). The eIF-2 can thus be considered the aminoacyl-tRNA binding factor for chain initiation. Factor eIF-1 may be necessary for the stabilization of the resulting complex, and evidence for a number of other protein co-factors that affect the ternary complex and their interactions has been reported (Dasgupta et al. 1976; DeHaro et al. 1978).

After the [40S•Met-tRNA$_f$] preinitiation complex has been formed, the binding of the mRNA to it is brought about by a family of proteins, including initiation factors eIF-4A, eIF-4B, eIF-4C, etc., which specifically recognize regions of the mRNA and the 40S preinitiation complex. It should be noted here that many eukaryotic mRNAs are known to have a 7-methyl-guanosine triphosphate (m^7-GTP) cap at the 5′-terminus (Both et al. 1975) and a sequence of poly(A) of varying lengths at the 3′-terminus (Darnell et al. 1971; Edmonds et al. 1971; Kates 1970; Lee et al. 1971; Lim and Canellakis 1970). In the newly formed preinitiation complex (lower right), both Met-tRNA$_f$ and mRNA are correctly placed on the 40S subunit, allowing for the formation of complementary base pairs between the anticodon on the tRNA$_f^{Met}$ and the initiation codon on mRNA. Finally, in the presence of initiation factor eIF-5, the large (60S) ribosomal subunit joins the 40S complex to form the 80S initiation complex (lower left), which is devoid of initiation factors. It is apparent from this series of reactions that the decoding of the initiation (AUG) codon has taken place in a region that is subsequently to become the P site of the ribosome. The second codon to be translated (after AUG) is adjacent to the P site, in the ribosomal "A" or "acceptor" site, which will be responsible for decoding all of the subsequent codons to be translated.

Chain Elongation

This series of reactions, involved in the translation of the internal codons, is presented schematically in figure 3. The aminoacyl-tRNA binding factor for chain elongation, elongation factor EF-1, reacts with aminoacyl-tRNAs (other than Met-tRNA$_f$) and GTP to form ternary complexes that then bind to the ribosome, placing the appropriate aminoacyl-tRNA at the A site (upper right).

As a consequence of this binding reaction, as in other reactions requiring GTP in protein synthesis, GTP is hydrolyzed to guanosine diphosphate and inorganic monophosphate. When the two aminoacylated tRNAs are adjacent to each other, one at the P site and the other at the A site, a ribosomal activity on the 60S subunit (peptidyltransferase) catalyzes the formation of a peptide bond. In the example shown in figure 3, a nucleophilic attack of the amino nitrogen of the incoming aminoacyl moiety at the A site on the carbonyl carbon of the methionyl moiety at the P site results in the formation of a dipeptidyl-tRNA. The peptidyl-tRNA, with methionine at the amino-terminal position, is attached to the tRNA of the incoming aminoacyl-tRNA at the A site (lower right). The peptidyltransferase reaction does not require a soluble translational protein factor or a nucleotide. Before the next codon can be translated, it must be moved to the A or decoding site of the ribosome. This reaction, which allows the movement of mRNA and of the ribosome in relation to each other, is accomplished by the translocation factor EF-2 and GTP; in the process, the deacylated $tRNA_f^{Met}$ at the P site is released, the peptidyl-tRNA is translocated to the ribosomal P site, a new codon is shifted into the A site, and GTP is hydrolyzed. The ribosome with peptidyl-tRNA at the P site and a new codon at the open A site (lower left), is now ready to repeat this series of chain-elongating reactions as shown below.

Figure 4 shows the series of reactions described above as they apply to the elongation of nascent chains. When a peptidyl-tRNA is at the P site and a new codon is available at the open A site (upper left), the following steps occur: binding of the appropriate aminoacyl-tRNA (filled circle, upper right) with EF-1 and GTP; formation of a peptide bond by peptidyltransferase; and translocation of the mRNA and the newly formed peptidyl-tRNA (one amino acid longer) from the A to the P site with EF-2 and GTP.

Chain Termination

When all of the meaningful codons in the mRNA have been translated, the final translocation step produces a complex in which the peptidyl-tRNA and the codon specifying the C-terminal amino acid are on the P site, and a termination codon is available at the open A site, as shown in the upper right part of figure 5. Normally, there is no tRNA with an anticodon sequence that can

form complementary base pairs with the termination triplet; under these specific conditions, protein factor RF (release factor) interacts with that A site in the presence of GTP. When the peptidyl-tRNA is at the P site and RF is at the A site, a peptidyltransferase reaction occurs, the completed polypetide chain is transferred to the elements of water and is then released from the ribosome. A subsequent series of reactions results in the release of the terminal tRNA and the ribosome (or subunits) from mRNA. The released ribonucleoprotein particles enter the free ribosome-subunit pool and participate in the initiation sequence of reactions leading to translation of mRNA. Thus, a ribosome cycle exists in which ribonucleoprotein particles are used catalytically for the translation of mRNAs and are converted from free subunits to 80S initiation complexes (ribosomes), then to polysomes, and eventually back to ribosomes and subunits.

A Cell-Free System for Translation of mRNA

To study the effects of various molecules on protein synthesis and on the intermediary steps involved in that process, it was essential that cell-free systems be developed capable of translating exogenous natural mRNA—systems that would permit examination of the effects on initiation, elongation, termination, etc. Several such cell-free systems have been prepared from cells that occur in the form of free-cell suspensions, such as reticulocytes, ascites, wheat germ, yeast, cultured mammalian cells, and so forth (Crystal et al. 1974; Fischer et al. 1980; Gasior et al. 1979; Marcus et al. 1974; Mathews and Korner 1970; Villa-Komaroff et al. 1974). With some exceptions (Heywood and Rourke 1974), similar active cell-free systems from organs of various animals, such as liver, muscle, and brain, are not currently available, although some of the preparations that have been described, for example, from rat liver (Burke et al. 1973; Moldave et al. 1979) appear to be encouraging. Thus, the present state of the art in eukaryotic protein synthesis does not permit the quantitative analysis of various intermediary reactions in translation, particularly of initiation reactions, in preparations from mammalian organs.

An example of the type of translational system that could be useful for the study of affectors of eukaryotic protein synthesis is the cell-free system developed in this laboratory from cultured

Chinese hamster ovary (CHO) cells (Fischer et al. 1980). As with most other preparations, it consists of an extract prepared by homogenizing cells and centrifuging the homogenate at 30,000 g for 20 minutes (figure 6) in order to remove whole cells, debris, nuclei, and mitochondria, leaving aminoacyl-tRNA synthetases, tRNAs, translational factors, polysomes, and ribosomes in the supernatant (S-30); the supernatant is chromatographed on a molecular sieve Sephadex column to remove low-molecular-weight components such as amino acids and nucleotides. When the S-30 fraction is incubated with a radioactive amino acid, an amino acid pool, nucleotides (ATP and GTP), and an energy-generating system (creatine phosphate and creatine phosphokinase), the isotopic amino acid is extensively incorporated into protein in the absence of added mRNA. Under these conditions, the endogenous mRNA in the polysomes in the preparation is translated. When the postmitochondrial fraction is treated with calcium and the Ca^{++}-dependent micrococcal nuclease, to degrade endogenous mRNAs (Pelham and Jackson 1976), and the nuclease is then inactivated by chelating the Ca^{++} with EGTA, the polysomes are completely degraded to ribosomes and ribosomal subunits, and the preparation (S-30′) now requires, in addition to the components described above, exogenous mRNA to carry out protein synthesis.

The requirements and the activity of the nuclease-treated CHO postmitochondrial fraction are shown in table 1. In the complete system, including globin mRNA and the components mentioned above, more than 12 pmol of radioactive amino acids are incorporated into protein; the extent to which radioactive amino acid is incorporated is markedly less in the absence of added mRNA (1 percent), nucleotides (6 percent), nucleoside triphosphate-generating system (2 percent), or an amino acid pool (4 percent). Figure 7 shows that protein synthesis in the presence of added mRNA (globin mRNA) is almost linear for up to 3 hours.

An interesting property of this cell-free preparation is that it is capable of synthesizing in vitro, with mRNA extracted from CHO cells, almost all of the proteins that are made in the intact cell. Figure 8 shows one-dimensional SDS-gel electrophoretic patterns of proteins extracted from (A) cells incubated with radioactive amino acid, and (B) proteins synthesized in vitro when nuclease-treated CHO extract is incubated with radioactive amino acid, all of the components required for protein synthesis, and RNA extracted from CHO cells (Krystosek et al. 1975) and enriched for poly(A)-containing mRNA by chromatography on poly(dT)-cellulose (Aviv

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and Leder 1972). Examination of these two patterns reveals more than 60 polypeptide bands that, with two exceptions marked by the arrows, appear to be identical; these results suggest that the cell-free system is capable of translating accurately all of the transcripts in CHO cells, and that it is able to carry out essentially all of the appropriate posttranslational modifications. It also should be noted that very large polypeptides, of molecular weight over 200,000, appear to be synthesized in vitro.

A question that could be asked with the system described here concerns the effect of alcohol on the protein synthesizing apparatus. An experiment designed to show the overall effect of varying concentrations of ethanol on the translation of endogenous and exogenous mRNAs is presented in figure 9. In one set of reactions (closed circles), the CHO postmitochondrial extract, prior to nuclease treatment, was incubated with radioactive amino acid and all the other components required for protein synthesis but not added mRNA; in another set of reactions (open circles), the nuclease-treated postmitochondrial extract was incubated with the same components plus globin mRNA. In both cases, translation was markedly inhibited when the alcohol concentration was about 2 percent or higher; at lower concentrations, in 1 percent alcohol, the translation of globin mRNA appeared to be inhibited to a slightly greater extent, suggesting that perhaps some reaction or reactions involved in chain initiation were more sensitive.

Figure 1. Phases of Protein Synthesis: Amino Acid Activation, Chain Initiation, Elongation, and Termination

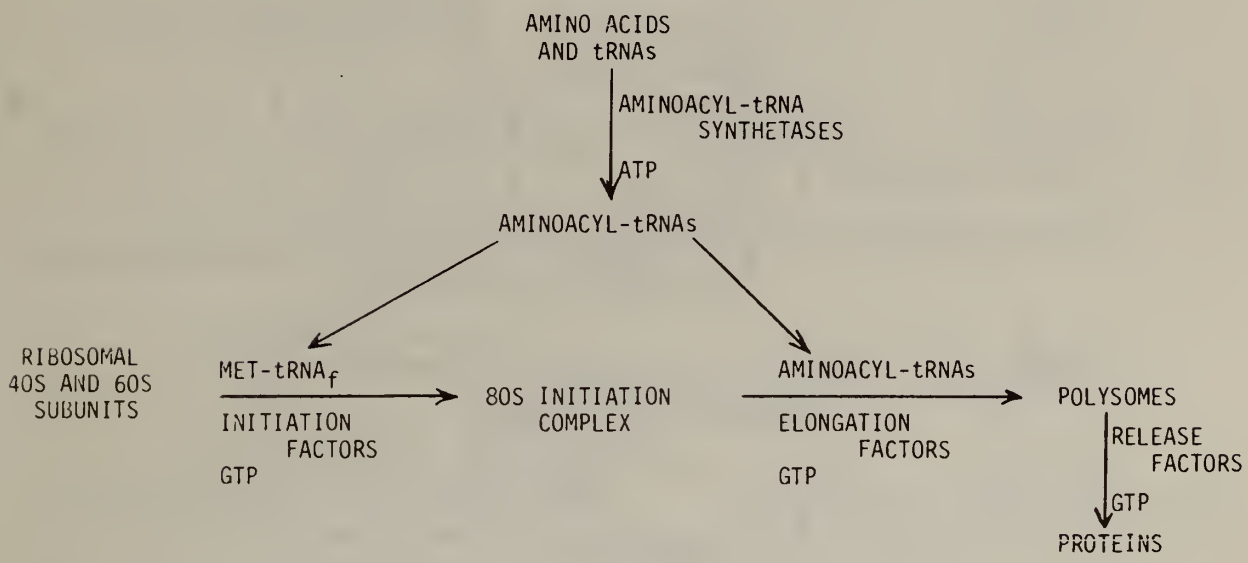


Figure 2. Scheme for Polypeptide Chain Initiation

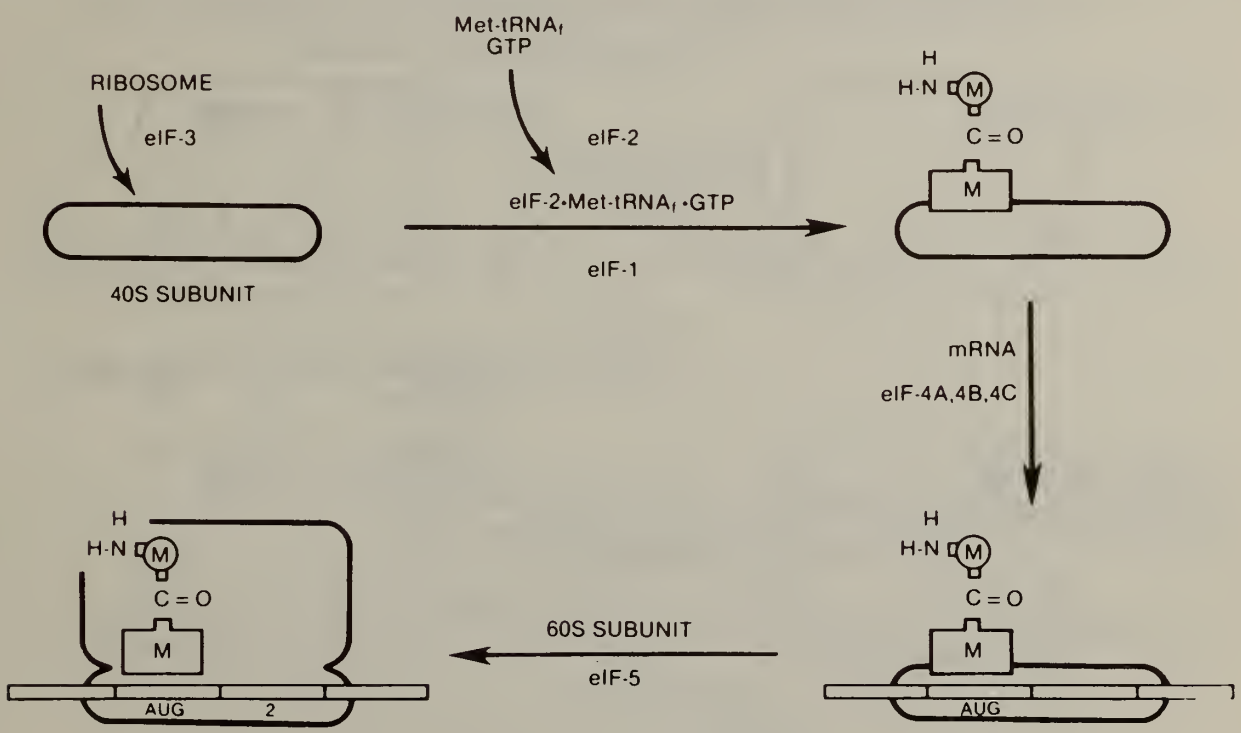


Figure 3. Scheme for Chain Elongation Reactions With the 80S Initiation Complex

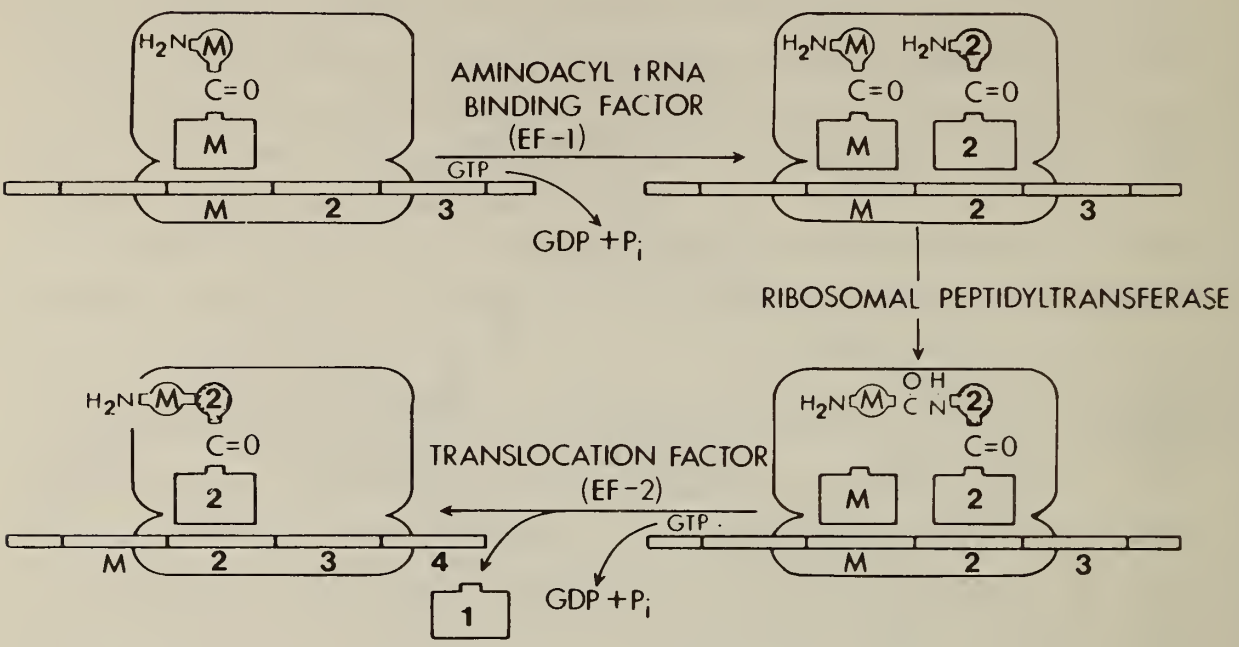


Figure 4. Elongation Reactions With Nascent Peptidyl Chains

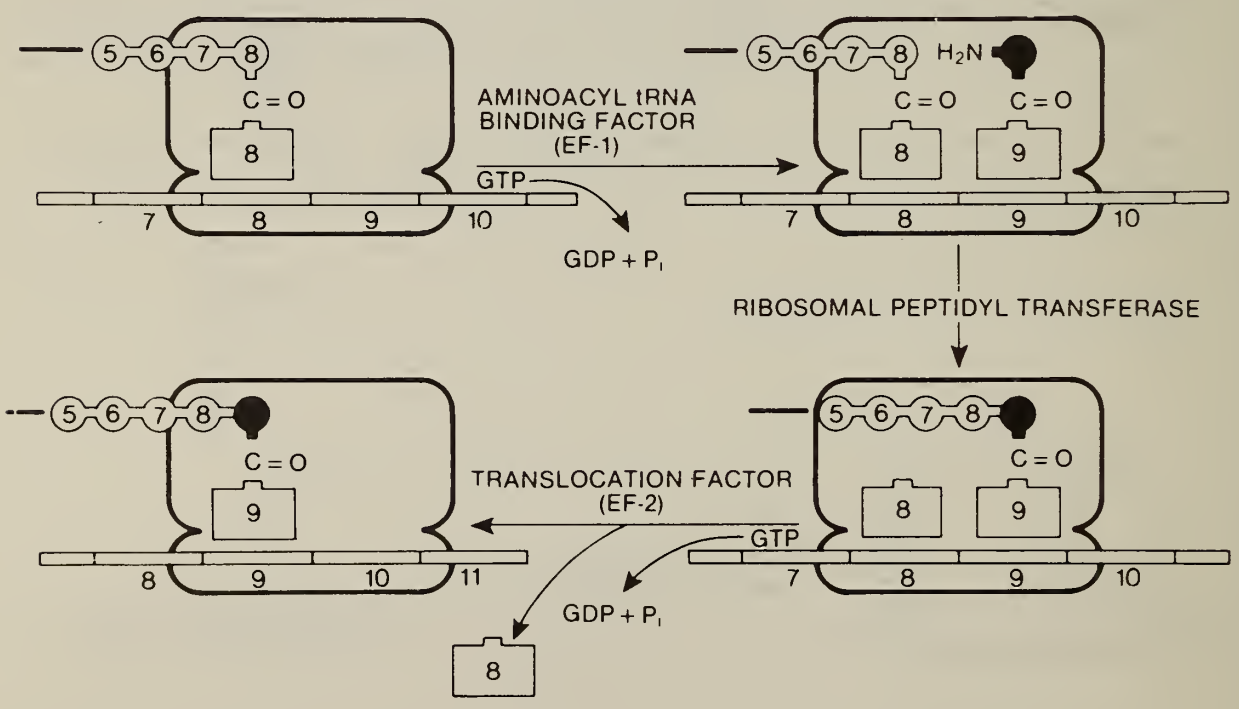


Figure 5. Scheme for Chain Termination

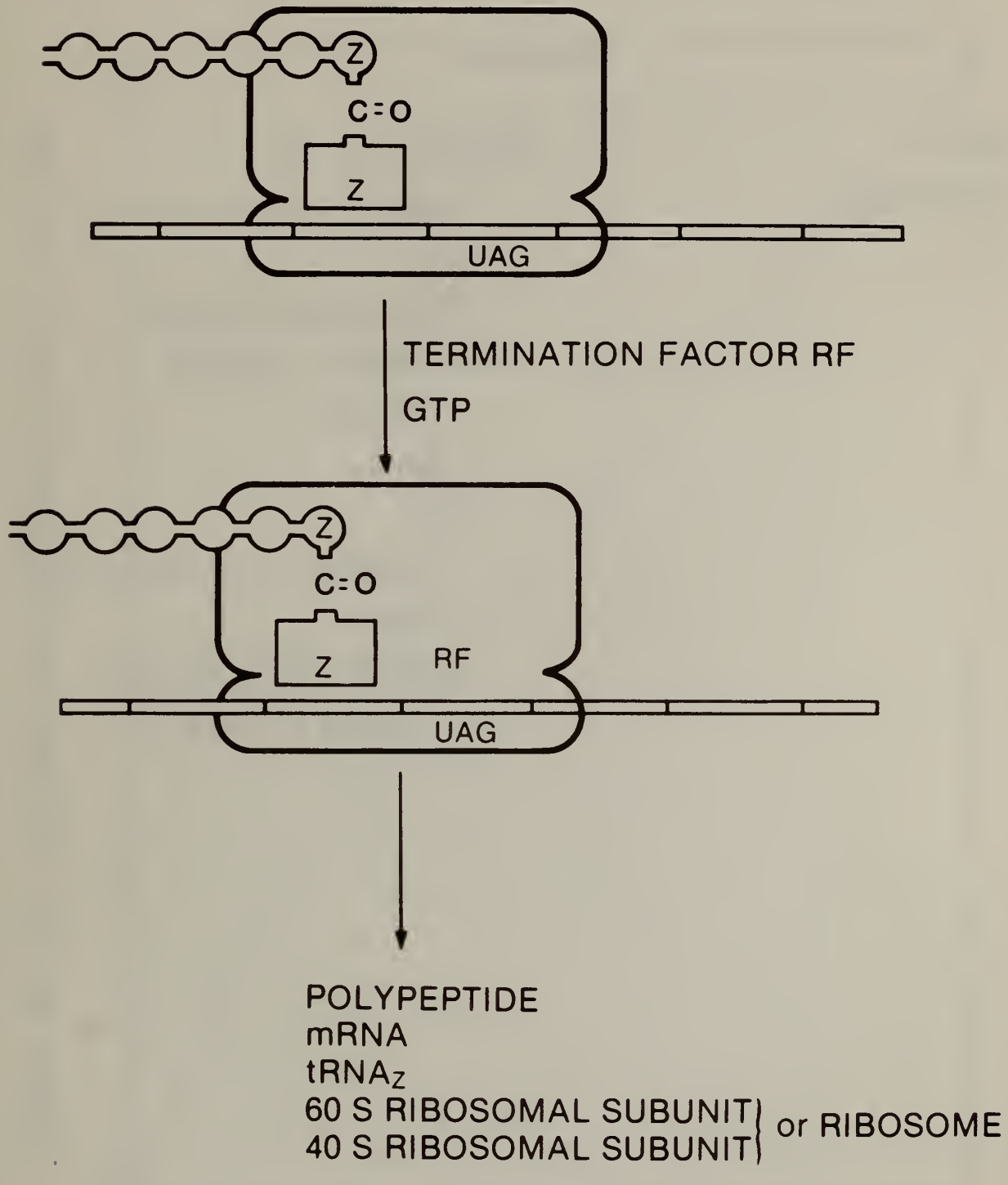


Figure 6. Scheme for Fractionation of Homogenates of Cultured Chinese Hamster Ovary Cells

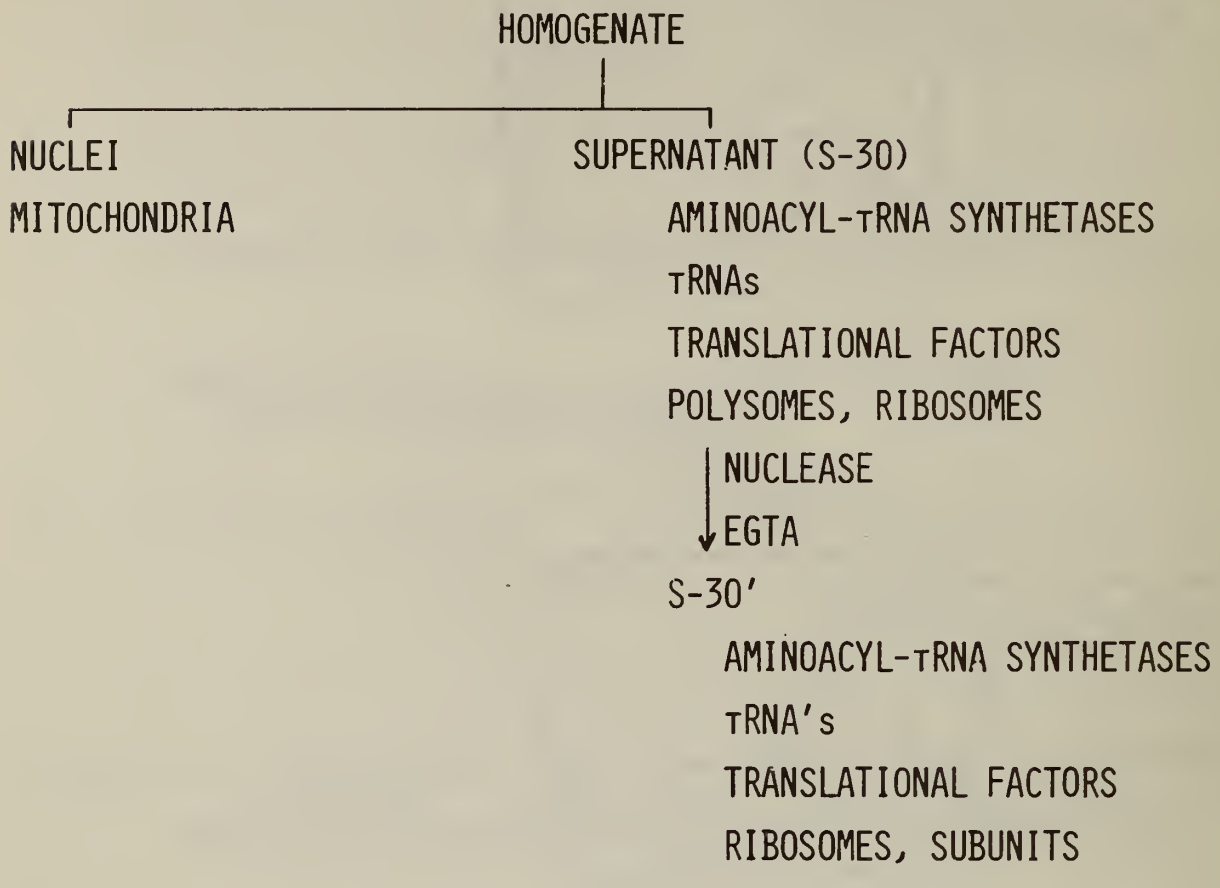


Figure 7. The Effect of Time of Incubation on the Translation of Globin mRNA With Nuclease-Treated Postmitochondrial CHO Extract

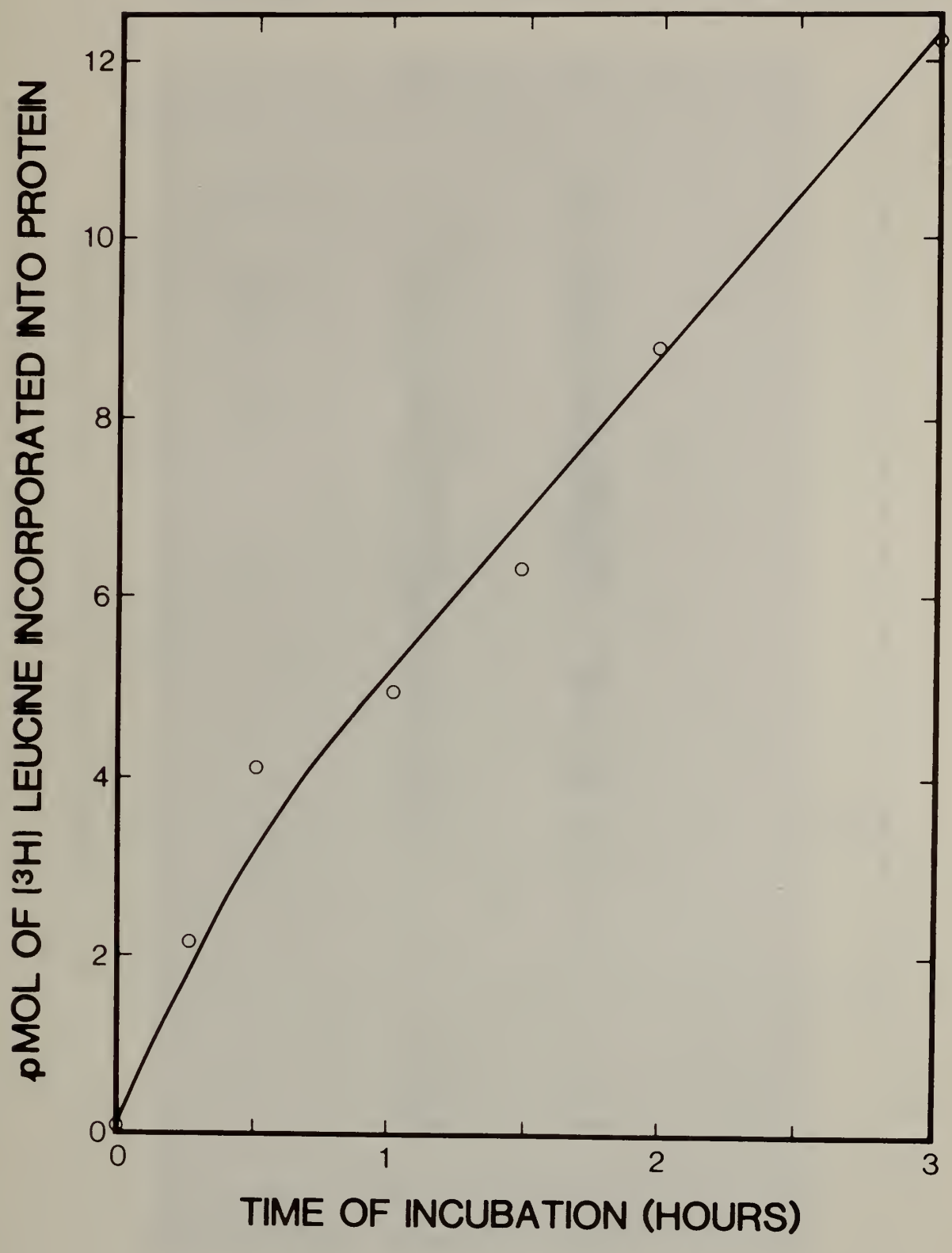
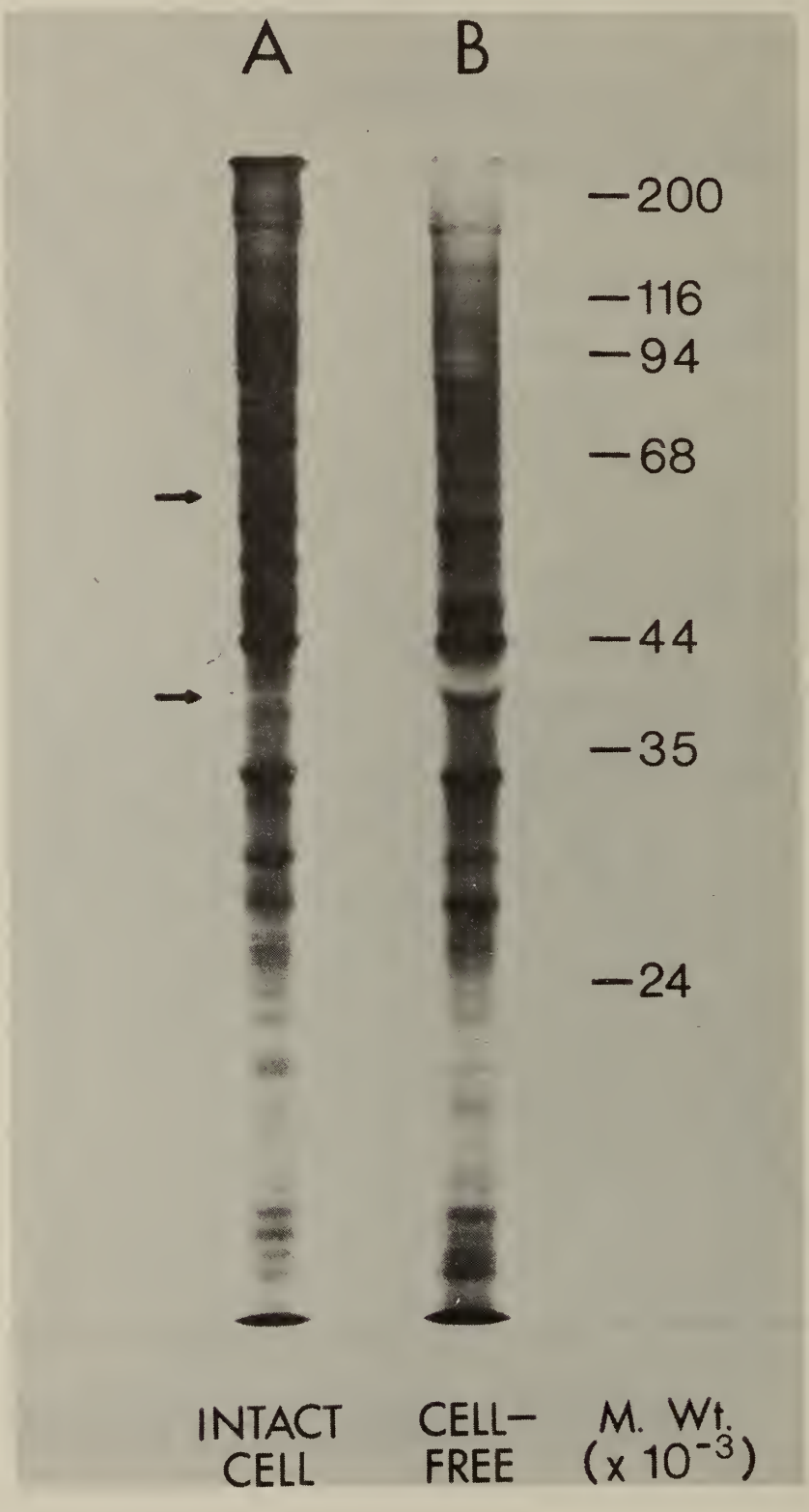


Figure 8. Gel Electrophoretic Analysis of the Products Synthesized in the Presence of (^{35}S)Methionine in (A) Intact Cells, and (B) the mRNA-Depleted Extract Plus Poly(A)$^+$ RNA From CHO Cells



SOURCE: Moldave and Fischer, *Analytical Biochemistry,* 113(1):13-26, 1981. Copyright 1981 by Academic Press.

Figure 9. The Effect of Varying Concentrations of Ethanol on the Translation of Endogenous Templates (S-30 Extract, No Added mRNA, Closed Circles), and of Exogneous Template (S-30′ Nuclease-Treated Extract, Plus Globin mRNA, Open Circles)

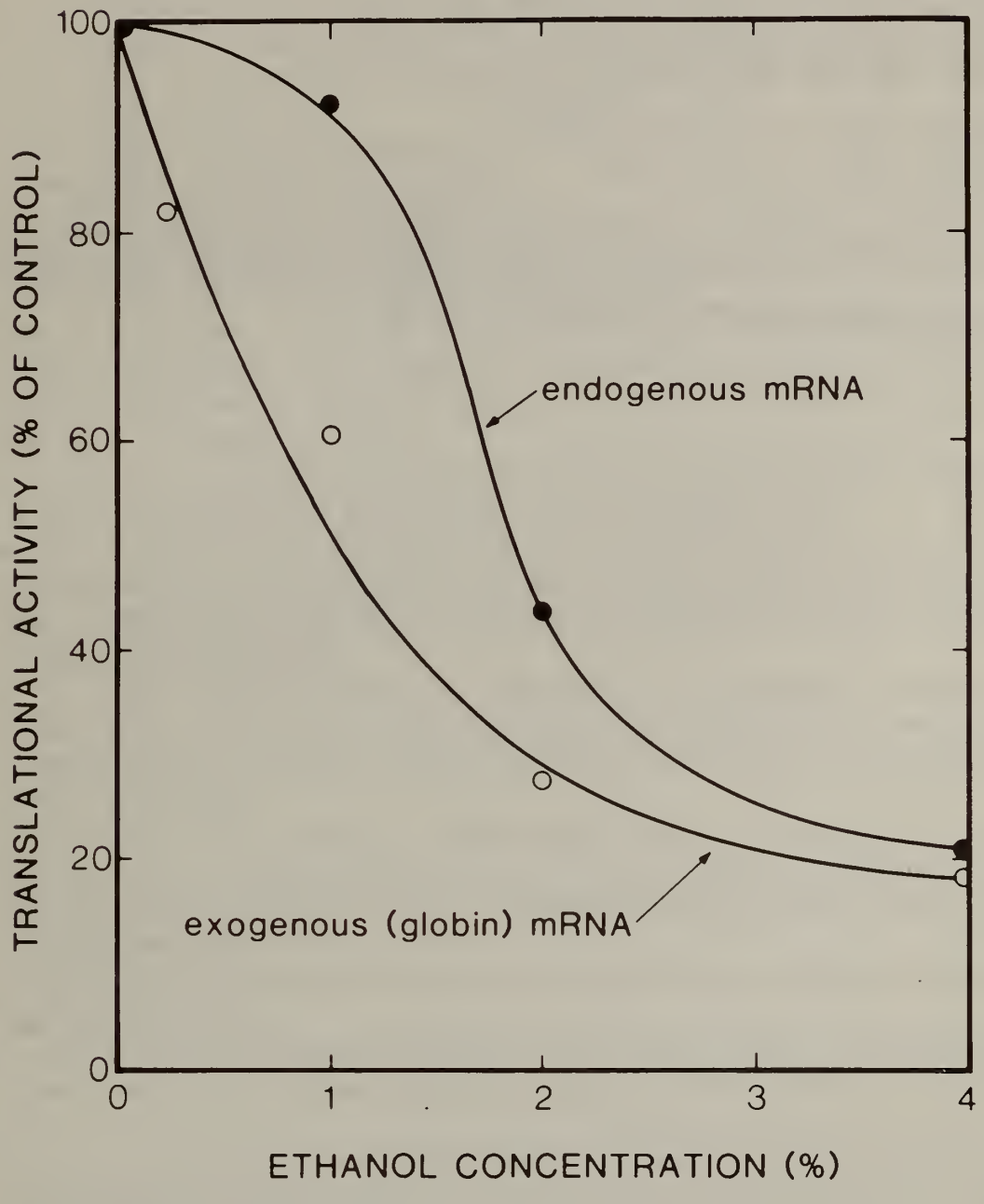


Table 1. Effect of Various Incubation Components on Protein Sythesis in a Cultured Cell (CHO) Extract Depleted of mRNA

Incubation Components	pmol of (^{3}H)Leucine Incorporated into Protein
Complete system[a]	12.23
Complete system, - mRNA	0.15
Complete system, - ATP and GTP	0.72
Complete system, - creatine phosphate	0.28
Complete system, - amino acids	0.48

[a] The complete system contained micrococcal nuclease-treated postmitochondrial extract, (^{3}H)leucine, globin mRNA, and all the other components as described previously (Fischer et al. 1980).

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Effects of Ethanol on Hepatic Protein Metabolism*

Enrique Baraona and Charles S. Lieber

Abstract

Alcohol consumption produces enlargement of the liver, which is caused by accumulation of proteins (in addition to fat and water) in the hepatocyte. The accumulated protein includes proteins normally destined for export into the plasma, such as albumin and transferrin. The increase in liver proteins could be due to increased synthesis, decreased secretion, or a combination of both mechanisms. The effects of ethanol on hepatic protein synthesis were studied in vivo and in vitro. Administration of ethanol (3 g/kg) in vivo produced no significant effects on synthesis of either total liver protein or albumin in naive rats and increased rather than decreased synthesis in animals chronically fed alcohol-containing diets. By contrast, in isolated hepatocytes, concentrations of ethanol similar to those found in vivo inhibited protein synthesis. However, the ethanol-induced inhibition observed in hepatocytes was associated with striking changes in ethanol metabolism compared to in vivo: reduced rate of ethanol oxidation, exaggerated shift of cytosolic redox state, and pyruvate depletion. Correction of these changes prevented the inhibitory effect of ethanol on hepatocyte protein synthesis. In contrast with the lack of significant in vivo effects of ethanol on protein synthesis (including those destined for export), the secretion of newly labeled albumin and transferrin from the liver into the plasma was significantly delayed, with retention of these proteins in the liver. The secretory alteration induced by ethanol was associated with disruption of liver microtubules and decreased polymerized tubulin, their major chemical component. The alteration of microtubules, an organelle the integrity of which is required for normal protein secretion, was reproduced by acute ethanol administration. This effect was partly prevented by decreasing the rate of ethanol oxidation with 4-methylpyrazole and

NOTE: Figures and table appear at end of paper.

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was aggravated by decreasing the rate of acetaldehyde oxidation with disulfiram. Chronic alcohol consumption exaggerates the effects of ethanol on microtubules by increasing the rate of ethanol oxidation and the accumulation of acetaldehyde in the liver. Acetaldehyde binds to tubulin, competes with colchicine for a similar binding site, and inhibits in vitro polymerization of tubulin. In conclusion, ethanol inhibits protein secretion from the liver, an effect that is mediated at least in part by acetaldehyde, a metabolite of ethanol capable of binding tubulin and of inhibiting its assembly into microtubules. In isolated hepatocytes, ethanol also inhibits protein synthesis, but the in vivo relevance of this effect remains to be determined.

One of the earliest and most striking manifestations of alcoholic hepatic damage is the enlargement of the liver. This hepatomegaly was reproduced in rats fed nutritionally adequate liquid diets containing 36 percent of total calories as ethanol. The increase in liver dry weight was due not only to the accumulation of fat but also to a significant increase in protein, compared with littermates pair-fed similar diets in which ethanol was isocalorically replaced either by additional carbohydrate or by fat (figure 1). Moreover, the protein accumulation included proteins normally destined for export into the plasma, such as albumin and transferrin (Baraona et al. 1975, 1977). The increase in liver volume was accounted for by an increase in size (rather than in number) of hepatocytes.

Effects of Ethanol on Hepatic Protein Secretion

To study the mechanism of the hepatic protein accumulation, we injected (^{14}C)leucine intravenously to both alcohol-fed and pair-fed control rats 90 minutes after intragastric administration of their corresponding diets, and we followed the time course of incorporation of this pulse labeling into plasma and hepatic protein and amino acid pools. The specific activity of the tracer was diluted to a greater extent in the alcohol-fed rats, already in the plasma and, even more, in the free amino acid and aminoacyl-tRNA pools of the liver (figure 2). This was due to an increase in leucine and other branched chain amino acids in plasma and liver of alcohol-fed rats, an effect also reported in alcohol-fed baboons (Shaw et al. 1978).

Despite the greater isotopic dilution, (^{14}C)leucine incorporation into immunoreactive albumin or transferrin of the liver was not decreased in alcohol-fed rats, at a time (10 minutes) when these newly labeled proteins were not yet significantly degraded or secreted into the plasma (figure 3). A similar lack of difference was

observed in the incorporation of (^{14}C)leucine into total liver proteins (at either 10 or 30 minutes after injection). If expressed per total liver and corrected by the isotopic dilution, these data indicate enhanced rather than decreased protein synthesis.

At later times, when active secretion of newly labeled protein into the plasma was taking place, there was a significant delayed appearance of these proteins in the plasma with a corresponding retention in the liver of the alcohol-fed rats (figure 3) (Baraona et al. 1977). We have found a similar secretory alteration after acute administration of ethanol (3 g/kg body weight) to naive chow-fed rats (Baraona et al. 1980).

Having established that alcohol impairs hepatic protein secretion, we focused on possible effects of ethanol on microtubules, a cytoskeletal organelle whose integrity is required for normal protein secretion (LeMarchand et al. 1973, 1974; Reaven and Reaven 1980; Redman et al. 1975, 1978; Stein and Stein 1973; Stein et al. 1974). To that effect, we first studied the acute effects of ethanol on liver microtubules both in rats chronically fed alcohol-containing diets and in their pair-fed naive controls. Microtubules were assessed morphometrically in electron micrographs, as described elsewhere (Matsuda et al. 1979), and biochemically by measuring the hepatic content of polymerized tubulin (the major chemical component of this organelle) according to the method of Pipeleers et al. (1977).

In the naive rats (pair-fed controls), intravenous infusion of ethanol decreased the volume density of microtubules (figure 4) and polymerized tubulin (1.41 $\pm$ 0.01 nmol/g of liver versus 1.74 $\pm$ 0.11 after infusion of saline; $p<0.01$). These decreases were partially prevented by methylpyrazole, an inhibitor of alcohol dehydrogenase. 4-Methylpyrazole itself did not affect microtubules.

In the rats previously fed alcohol, withdrawal from ethanol for 20 hours restored microtubules to values similar to those of controls. Rechallenges with intravenous ethanol decreased microtubules to a greater extent than in the pair-fed controls (figure 4). A similar decrease, though not so striking, occurred in polymerized tubulin (1.28 $\pm$ 0.04 nmol/g of liver versus 1.68 $\pm$ 0.08, after saline infusion; $p<0.01$). Moreover, the addition of 4-methylpyrazole failed to prevent most of the inhibitory effect of ethanol.

These results indicate that acute ethanol administration decreases liver microtubules, an effect that is mediated at least in part by ethanol oxidation through the alcohol dehydrogenase pathway. Furthermore, chronic alcohol consumption enhances this

effect, but this enhancement is mediated at least in part by a pyrazole-insensitive mechanism.

To investigate whether the enhanced toxicity of ethanol in alcohol-fed animals is due to differences in ethanol metabolism between the two types of rats, we measured hepatic ethanol and acetaldehyde concentrations in freeze-clamped livers (Ericksson et al. 1977) and calculated the rate of ethanol oxidation from the clearance of blood ethanol following intraveneous administration, with and without 4-methylpyrazole (table 1). Alcohol-fed rats oxidized ethanol at a faster rate than did controls, resulting in lower ethanol and higher acetaldehyde concentrations in the liver, at the time of the microtubule assessment. Inhibition of alcohol dehydrogenase activity by 4-methylpyrazole reduced the rate of ethanol oxidation by more than 70 percent. However, the residual rate and the acetaldehyde concentration in the liver were still higher in ethanol-fed rats than in their pair-fed controls. The dose of ethanol was decreased in experiments with 4-methylpyrazole in order to produce comparable ethanol concentrations at the time of microtubule assessment.

These results indicate that the induction of a pyrazole-insensitive pathway of ethanol oxidation (most likely microsomal) in alcohol-fed rats contributes to the enhanced toxicity of ethanol on microtubules by increasing generation of oxidation products such as acetaldehyde.

To determine whether the microtubular alteration was due to acetaldehyde itself or to its oxidation products, we gave disulfiram (300 mg/kg body weight by gastric tube 20 hours prior to sacrifice) to inhibit the oxidation of acetaldehyde to acetate by aldehyde dehydrogenases. After pretreatment with disulfiram, the infusion of ethanol markedly increased the hepatic concentration of acetaldehyde in both alcohol-fed and control rats (figure 5). The reduction of microtubules was more striking after the combination of ethanol and disulfiram than after ethanol alone. Disulfiram alone did not affect microtubules. After disulfiram, again rats pretreated chronically with alcohol had higher acetaldehyde concentrations and greater microtubular disruption than did the naive controls.

Since the decrease in polymerized tubulin induced by ethanol was associated with increased free tubulin (Matsuda et al. 1979), we investigated whether ethanol or its metabolites were capable of inhibiting tubulin polymerization in vitro. To this effect, we partially purified tubulin from rat brain and produced microtubules in vitro, according to the procedure of Shelanski et al. (1973).

The formation of these microtubules was followed by light scattering as described by Gaskin et al. (1974). Preincubation of 4 to 9 nmoles of tubulin/ml with 200 μM acetaldehyde in sealed cuvettes inhibited the in vitro formation of microtubules (figure 6). Ethanol (50 mM) or acetate (1 mM) did not have such an effect.

Thus, acetaldehyde inhibits tubulin polymerization in a way similar to colchicine, although to a much lesser degree. Since colchicine inhibits the assembly of microtubules because it specifically binds tubulin, we wondered whether acetaldehyde can compete with colchicine for this binding. To study this effect, we incubated liver cytosol with (^{3}H)colchicine in concentrations ranging from 1 to 5 μM and measured the amount of colchicine bound to tubulin by the method of Sherline et al. (1974). Multiple additions of acetaldehyde in order to maintain concentrations of 214 ± 15 μM inhibited the binding of colchicine to tubulin (figure 7). The inhibition was greater at the lowest colchicine concentrations, did not affect the maximal binding of colchicine, but changed the affinity of colchicine for tubulin. These findings indicate that acetaldehyde and colchicine compete for a similar binding site and suggest that the ability of acetaldehyde to inhibit tubulin polymerization may be due to its binding at a key site required for tubulin polymerization. This represents the most likely mechanism for the disruption of liver microtubules after alcohol administration. The microtubular damage can be responsible, at least in part, for the impairment of secretion and retention of export proteins in the liver. Altered secretion of glycoproteins (Sorrell and Tuma 1978; Sorrell et al. 1977) and albumin (Jennett et al. 1980) have also been found in rat liver slices incubated with ethanol. This secretory alteration in liver slices has been reproduced with acetaldehyde.

Effects of Ethanol on Hepatic Protein Synthesis

Contrasting with the alterations in secretion, significant inhibition of protein synthesis in the total liver in vivo was not found. After acute intragastric administration of liquid diets providing ethanol (3 g/kg body weight), the specific activity of (^{14}C)leucine into hepatic leucyl-tRNA and the incorporation into liver proteins were unaffected. As mentioned above, this acute treatment resulted in enhanced rather than reduced protein synthesis in the total liver

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of rats previously fed the alcohol-containing diets for several weeks. The mechanism of this stimulation of protein synthesis remains to be determined. The administration of ethanol in these experiments produced blood ethanol concentrations of 20 to 30 mM, which are the maximal concentrations we found in the rats spontaneously ingesting the liquid diets. The addition of ethanol, in concentrations ranging from 6 to 50 mM, to hepatocytes isolated from fed rats produced 20 to 30 percent inhibition of leucine incorporation into cell protein. The specific activity of intracellular leucine was not altered by ethanol, indicating that the differences in incorporation do indeed reflect differences in protein synthesis.

This discrepancy between the in vivo and in vitro effects of ethanol raised two major questions: (a) What is the mechanism of the effect of ethanol in vitro? (b) To what extent does this in vitro finding pertain to conditions prevailing in vivo?

The oxidation of ethanol by hepatocytes incubated in saline solutions and glucose is markedly decreased compared to in vivo conditions. This decreased rate of ethanol oxidation is associated with marked exaggeration of the shift in cytosolic redox state (Baraona et al. 1980). This has been attributed to relative inability of these cells to translocate reducing equivalents from the cytosol into the mitochondria because of depletion of substrates for the malic-aspartic acid shuttle (Crow et al. 1977, 1978).

To determine whether the inhibition of protein synthesis in hepatocytes was linked to the oxidation of ethanol, we studied the effects of 4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase (figure 8). The addition of 4-methylpyrazole did not alter significantly protein synthesis by hepatocytes. However, it did prevent most of the inhibitory effect of ethanol, indicating that under these conditions the inhibition is due to ethanol oxidation rather than to ethanol itself. At higher ethanol concentrations and particularly in hepatocytes from fasted rats, there are also inhibitory effects of ethanol on protein synthesis that are not prevented by 4-methylpyrazole (Morland et al. 1980).

The prevention by 4-methylpyrazole was associated with a 70 percent decrease in the rate of ethanol oxidation, which virtually abolished the accumulation of acetaldehyde and the shift in cytosolic redox state, as judged from the lactate/pyruvate ratio.

To investigate whether the inhibition of protein synthesis was mediated by acetaldehyde, we increased the rate of ethanol oxidation and the generation of acetaldehyde by removing reducing equivalents from the cytosol (figure 9) in two ways: (a) by adding an

artificial scavenger of reducing equivalents, such as methylene blue; and (b) by adding substrates for the malic-aspartic acid shuttle to facilitate the translocation of reducing equivalents from the cytosol into the mitochondria. In both situations, the rate of ethanol oxidation doubled and the accumulation of acetaldehyde increased tenfold. However, the ethanol-induced inhibition of protein synthesis was prevented rather than enhanced. Thus, acetaldehyde is not a likely mediator of this effect of ethanol, despite the fact that acetaldehyde concentrations of 320 μM or higher are capable of inhibiting protein synthesis in liver slices (Perin and Sessa 1975; Sorrell et al. 1977).

To investigate whether the ethanol-induced inhibition of protein synthesis was due to the highly reduced state of the cytosol, we studied the effects of either pyruvate or lactate supplementation (figure 10). The addition of pyruvate decreased the lactate/pyruvate ratio during ethanol oxidation and prevented the ethanol-induced inhibition of protein synthesis. However, the addition of lactate, which increased the lactate/pyruvate ratio even further, also prevented the inhibition of protein synthesis. Thus, it is also unlikely that the inhibition of protein synthesis is a direct consequence of the exaggerated shift in cytosolic redox state during ethanol oxidation. Therefore, we focused on other metabolic derangements induced by ethanol oxidation.

Ethanol oxidation in hepatocytes from fed rats was associated with marked depletion of pyruvate. Moreover, all of the agents that we showed do prevent the inhibition of protein synthesis. They also have a common characteristic of sparing pyruvate from complete depletion (figure 11). Furthermore, not only was pyruvate decreased, but also the sum of pyruvate plus lactate (the two major products of glycolysis). It is not likely that these decreases could be due to excessive consumption, because the two major pathways of utilization—gluconeogenesis and Krebs cycle activity—are known to be inhibited by ethanol. Pyruvate plays a central role in energy metabolism, and its depletion may divert amino acids from protein synthesis to the supply of fuel. In vivo, however, the administration of ethanol to rats results in a modest decrease in the concentration of pyruvate in the total liver, with only a twofold rise in the lactate/pyruvate ratio (Baraona et al. 1980; Lindros and Aro 1969).

Thus, the question remains whether these alterations occurring in isolated hepatocytes pertain to conditions prevailing in vivo.

Figure 1. Increased Liver Protein in Rats Fed Liquid Diets Containing 36 Percent of Total Calories as Ethanol for 4 to 6 Weeks, Compared With Littermates Limited to Consumption of Equal Amounts of Diets in Which Ethanol Calories Were Replaced by Either Carbohydrate or Fat

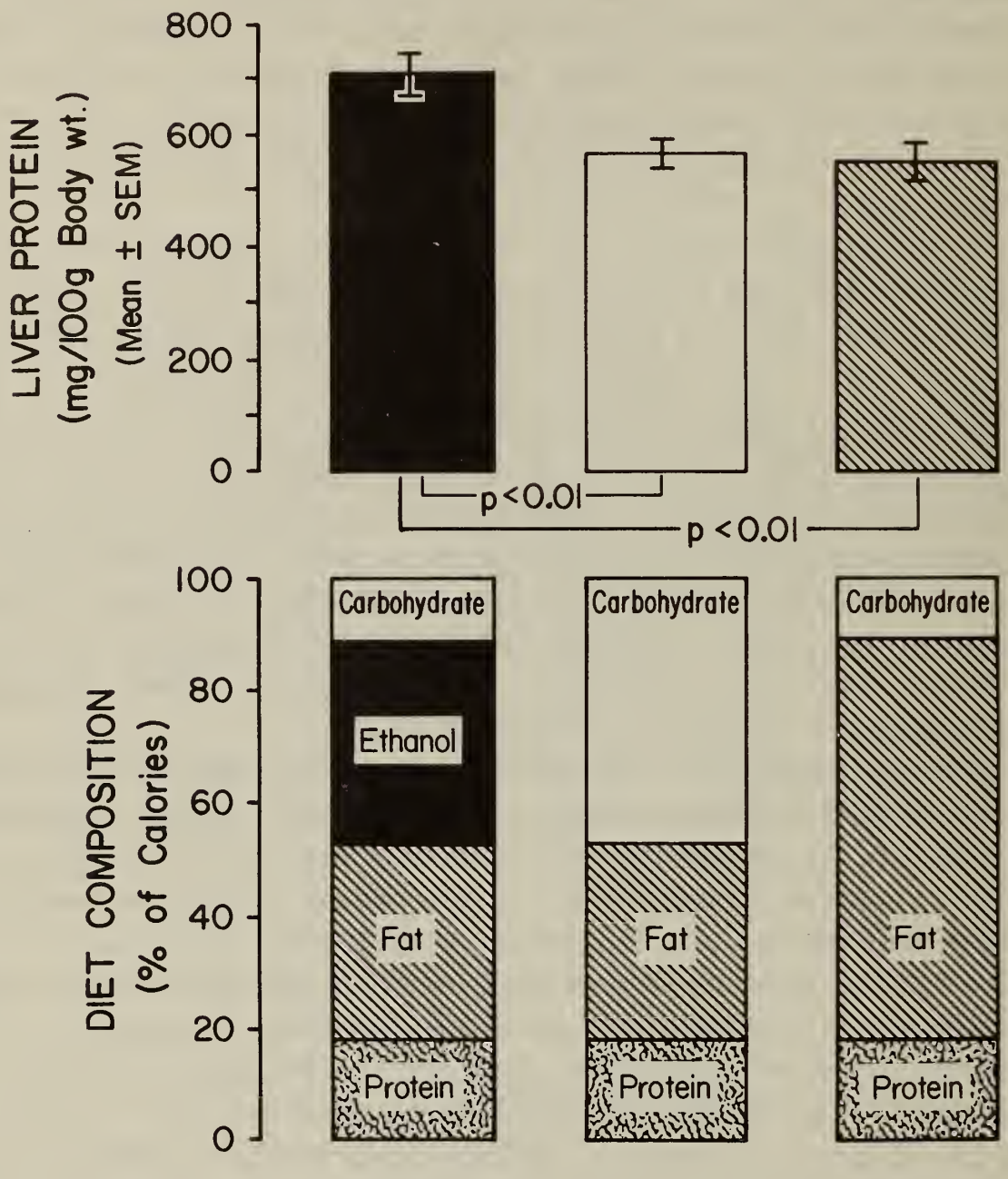
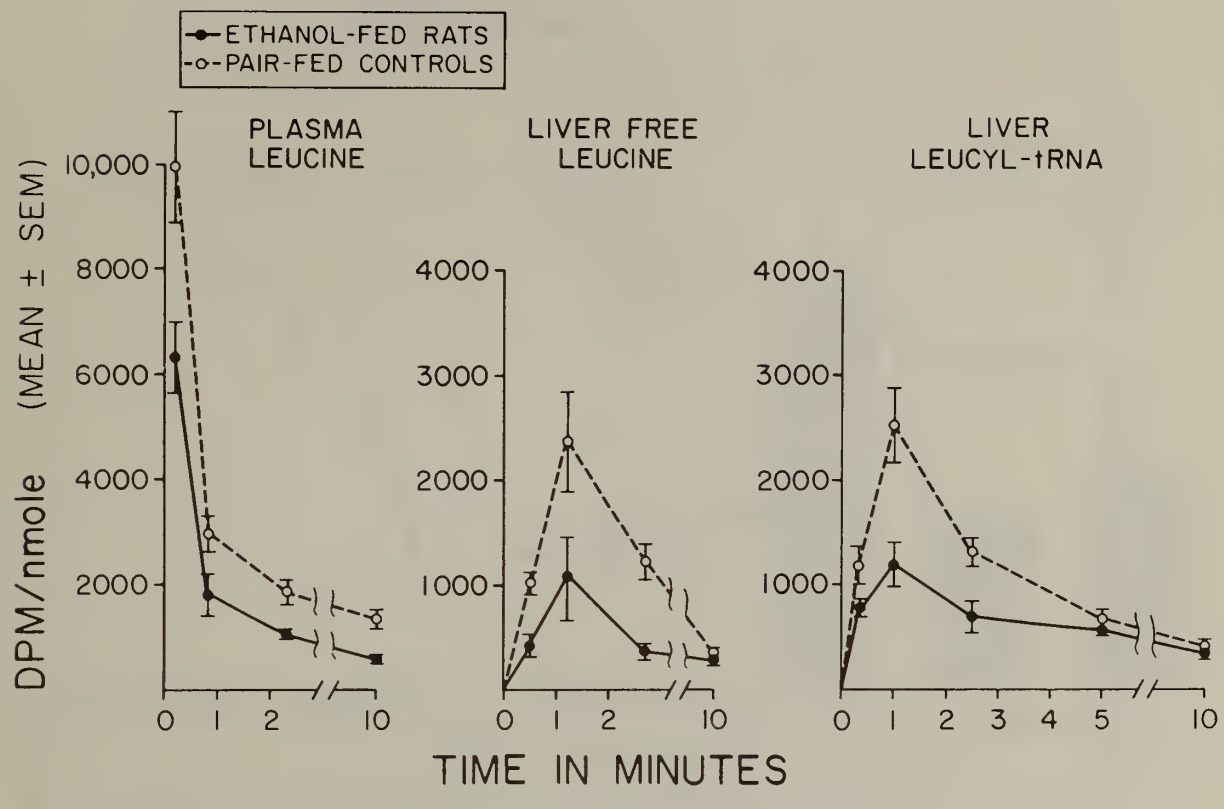
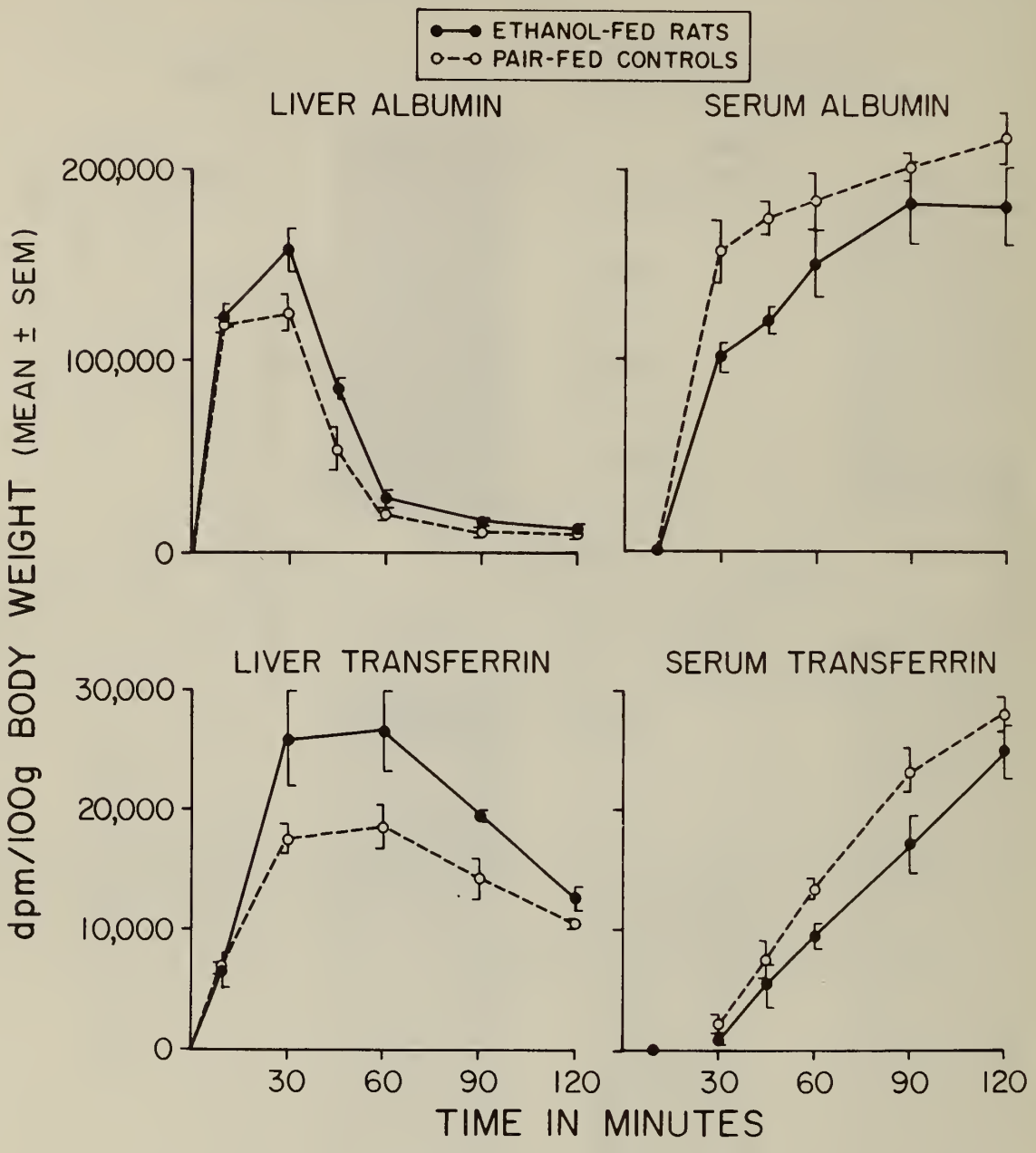


Figure 2. Specific Activities of Serum and Liver Leucine and Hepatic Leucyl-tRNA After Intravenous Injection of (^{14}C)Leucine to 23 Pairs of Rat Littermates Fed Either Ethanol-Containing or Control Diets for 4 to 6 Weeks



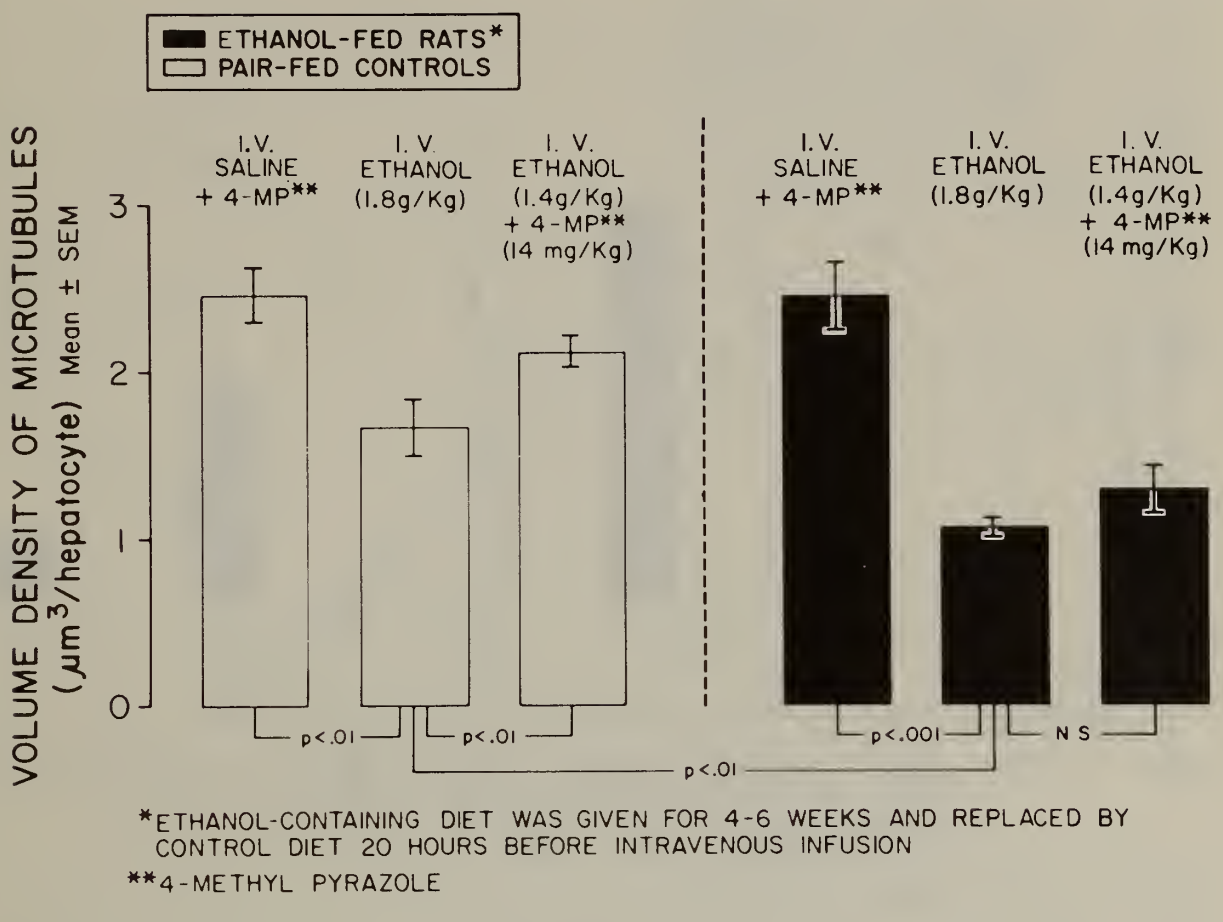
Note: Each point represents the average of two to five animals. Leucine-specific activities were lower in ethanol-fed rats compared with those in their pair-fed controls ($p < 0.02$; paired comparisons). The decreased specific activities were due to larger leucine pools in both serum and liver of ethanol-fed rats.

Figure 3. Incorporation of Intravenously Injected (^{14}C)Leucine Into Liver and Serum Albumin and Transferrin at Various Time Intervals in Rats Pair-Fed Either Ethanol-Containing or Control Diet for 4 to 6 Weeks



Note: Each point represents the average of three to six animals. Labeling of liver albumin (30 to 45 min after injection) and of liver transferrin (30 to 90 min) was significantly greater in ethanol-fed rats, whereas labeling of serum albumin and transferrin (same periods) was significantly reduced in these rats compared to controls ($p < 0.01$; paired comparisons). After 60 min in the case of serum albumin, and at 120 min in the case of transferrin, the differences in labeling between both types of animals were not statistically significant. The incorporation of (^{14}C)leucine into liver albumin (10 min) and transferrin (30 min) in ethanol-fed rats were similar or higher than in controls.

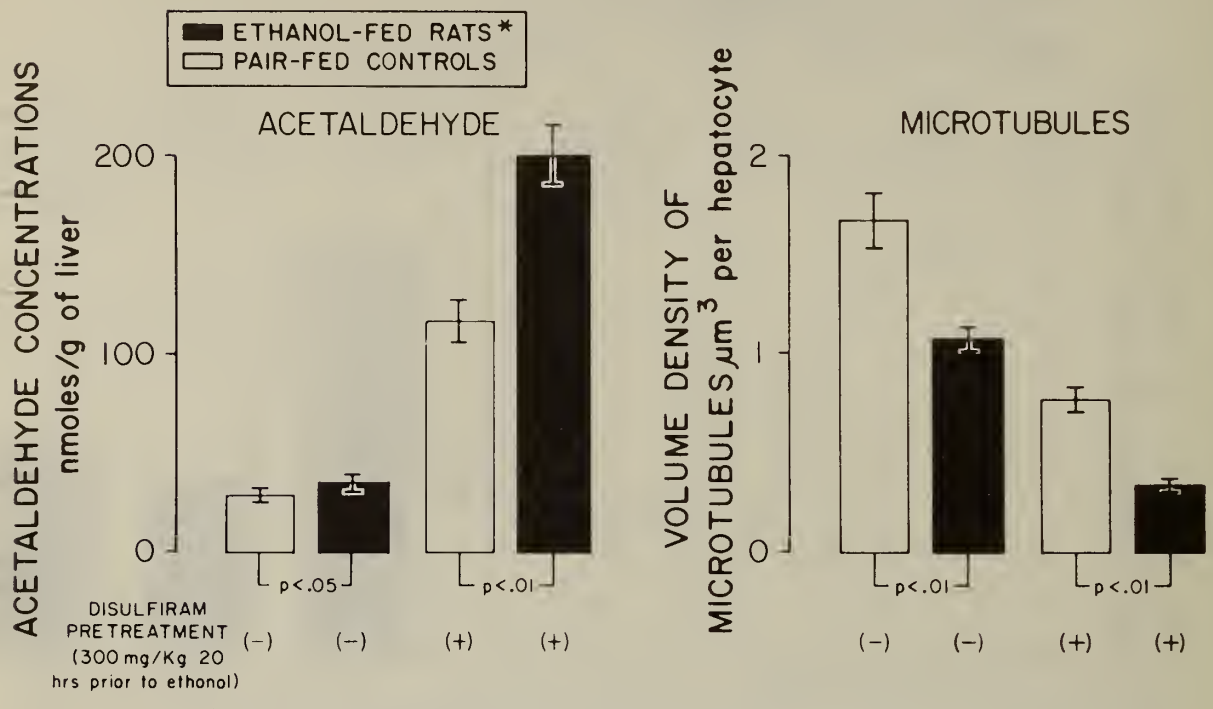
Figure 4. Acute Effects of Ethanol (With or Without 4-Methylpyrazole) on Liver Microtubules of Rats Pair-Fed Liquid Diets Containing 36 Percent of Calories as Either Ethanol or Isocaloric Carbohydrate for 4 to 6 Weeks



SOURCE: Baraona et al., in press.

Note: Acute ethanol administration decreased liver microtubules in both types of animals but to a greater extent in alcohol-fed rats. 4-Methylpyrazole partially prevented the inhibitory effect of ethanol especially in naive (pair-fed control) animals.

Figure 5. Acute Effects of Ethanol (With or Without Disulfiram) on Hepatic Acetaldehyde Concentrations and Microtubules of Rats Pair-Fed Liquid Diets Containing 36 Percent of Calories as Either Ethanol or Isocaloric Carbohydrate for 4 to 6 Weeks

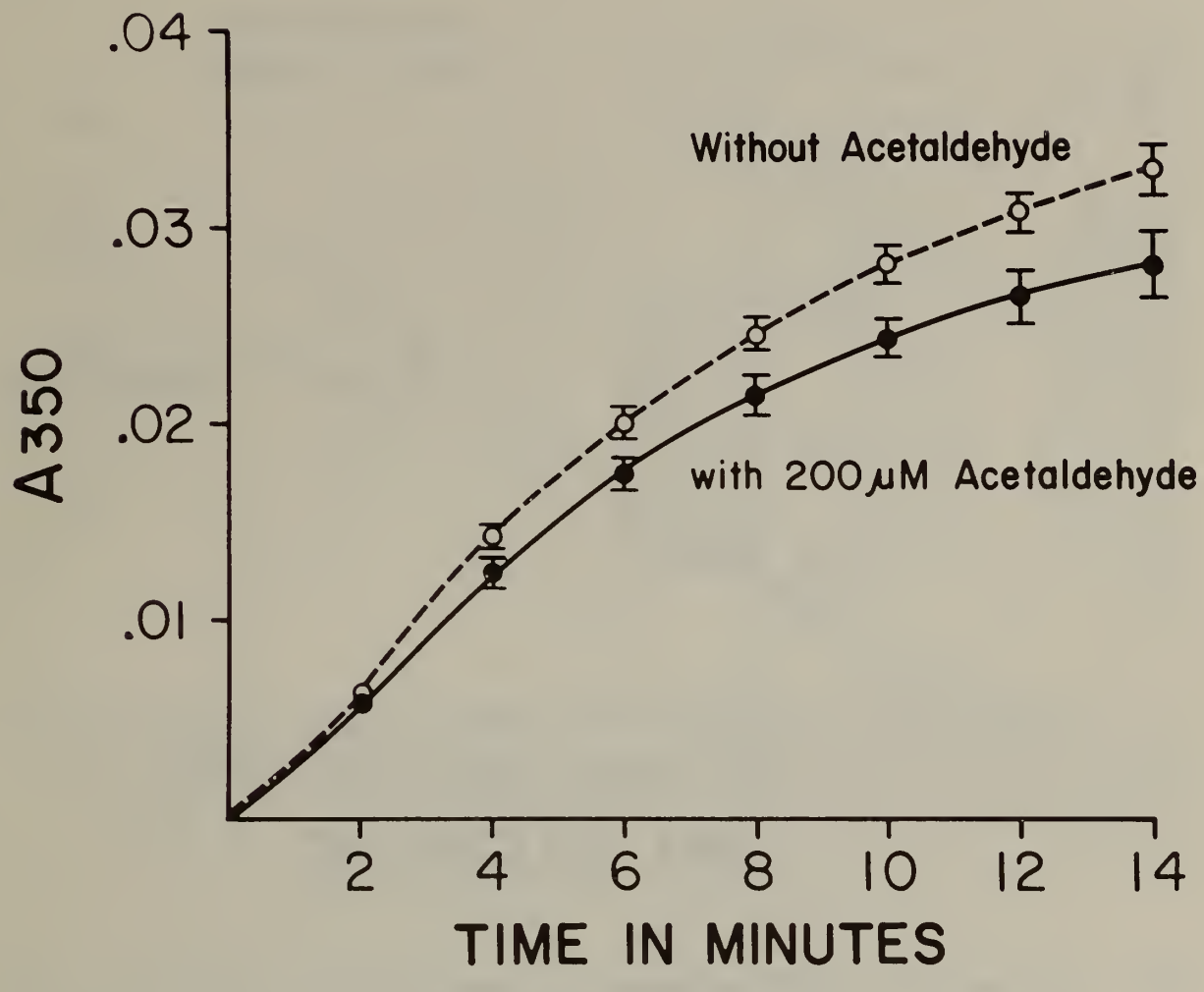


*ETHANOL-CONTAINING DIET WAS GIVEN FOR 4-6 WEEKS AND REPLACED BY CONTROL DIET 20 HOURS BEFORE I.V. ADMINISTRATION OF ETHANOL

SOURCE: Baraona et al., in press.

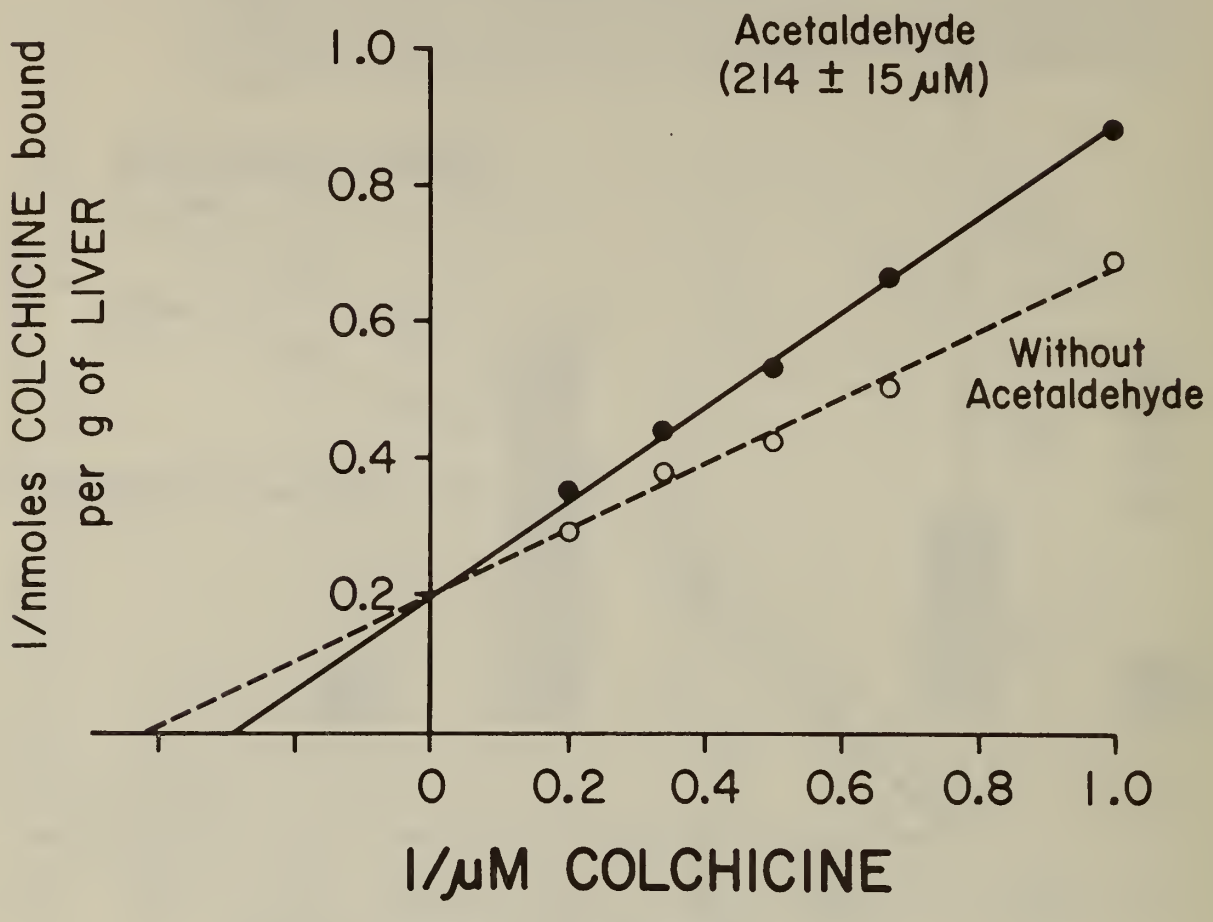
Note: Pretreatment with disulfiram increased the ethanol-induced accumulation of acetaldehyde and the decrease in microtubules, especially in alcohol-fed rats.

Figure 6. Inhibitory Effect of Acetaldehyde on In Vitro Polymerization of Tubulin



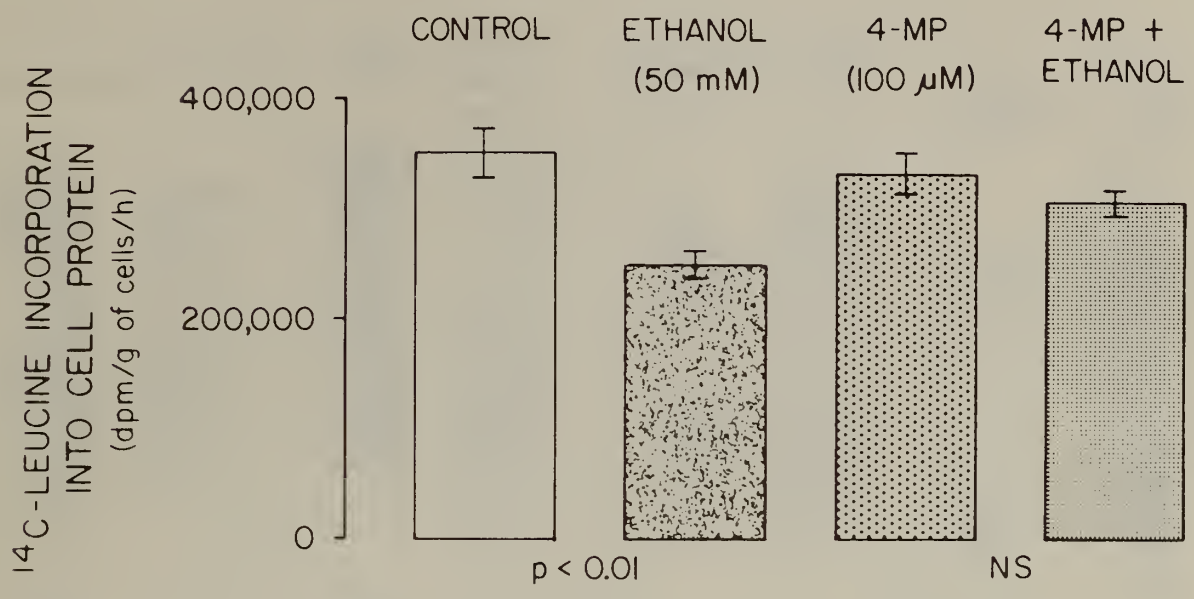
Note: Tubulin was prepared from rat brains by two cycles of polymerization-depolymerization (Shelanski et al. 1973) and diluted to concentrations (6 to 9 nmol/ml) comparable to that existing in liver. Polymerization rate at 30°C in the presence of GTP was assessed by the change in optical density at 350 mμ wavelength (Gaskin et al. 1974). 200 μM acetaldehyde decreased significantly ($p < 0.02$) the rate of tubulin polymerization.

Figure 7. Competitive Inhibition of Colchicine Binding to Liver Tubulin by Acetaldehyde



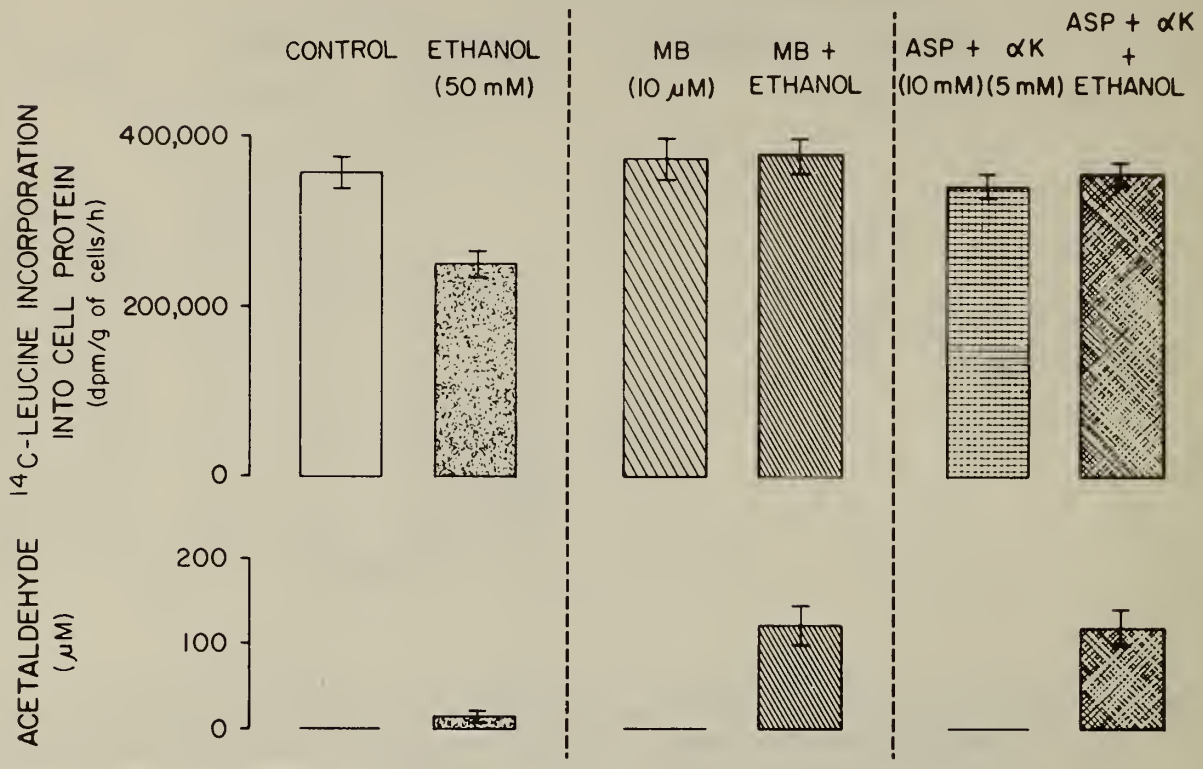
Note: The binding of (^{3}H)colchicine (in concentrations ranging from 1 to 5 μM) to liver cytosol containing 2 mM pyrazole was measured by the charcoal method of Sherline et al. (1974). At these low concentrations, colchicine binds specifically to liver tubulin (Patzelt et al. 1975). The effects of multiple additions of acetaldehyde to maintain stable concentrations in sealed incubation flasks were compared with that of equal volumes of water. 2 mM pyrazole was used to inhibit reduction of acetaldehyde to ethanol. The double reciprocal plot indicates that acetaldehyde inhibits colchicine binding, especially at the lower colchicine concentrations, does not affect the maximal binding, and decreases the apparent affinity of tubulin for colchicine.

Figure 8. Inhibition of Protein Synthesis in Isolated Hepatocytes Produced by Ethanol and Its Prevention by 4-Methylpyrazole



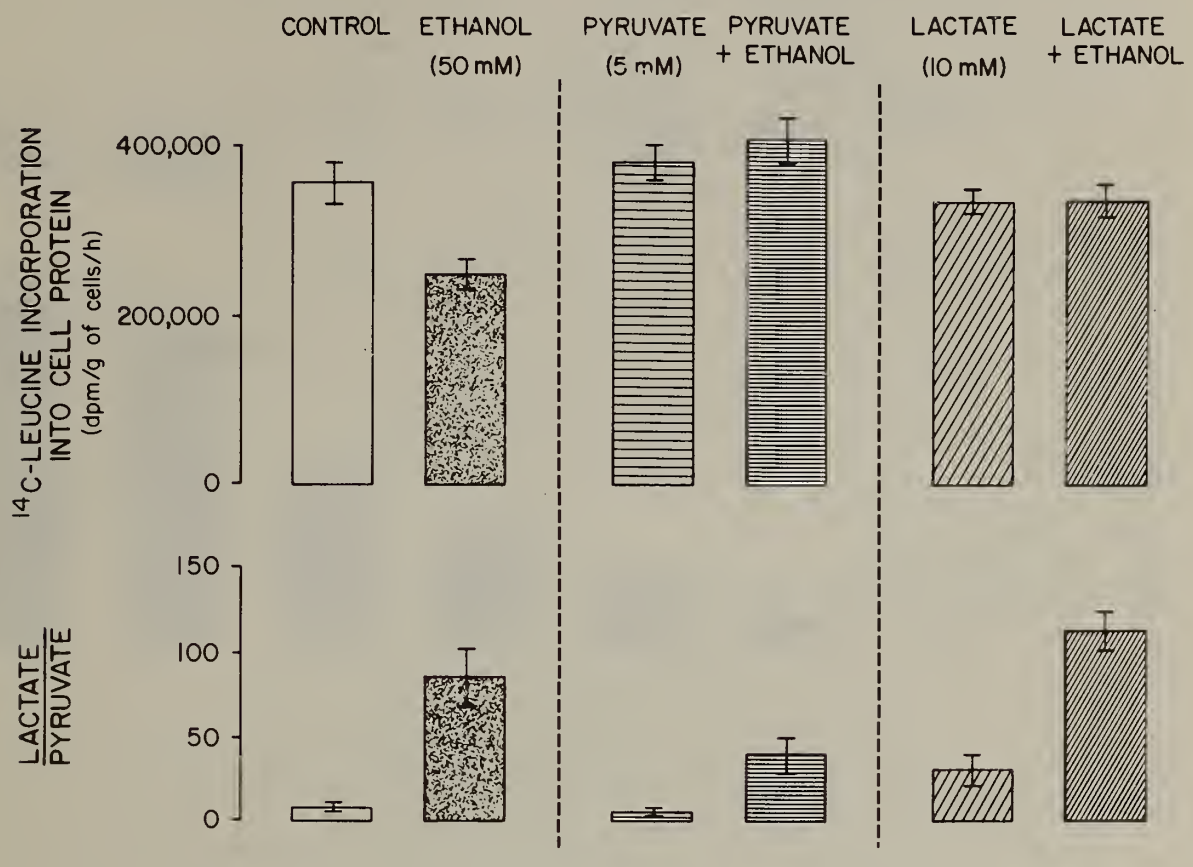
Note: The decreased incorporation of (^{14}C)leucine produced by ethanol most likely reflects inhibition of protein synthesis, because ethanol did not change the specific activity of leucine in the intracellular amino acid pools.

Figure 9. Relationship Between Acetaldehyde Concentrations and the Ethanol-Induced Inhibition of Protein Synthesis in Hepatocytes



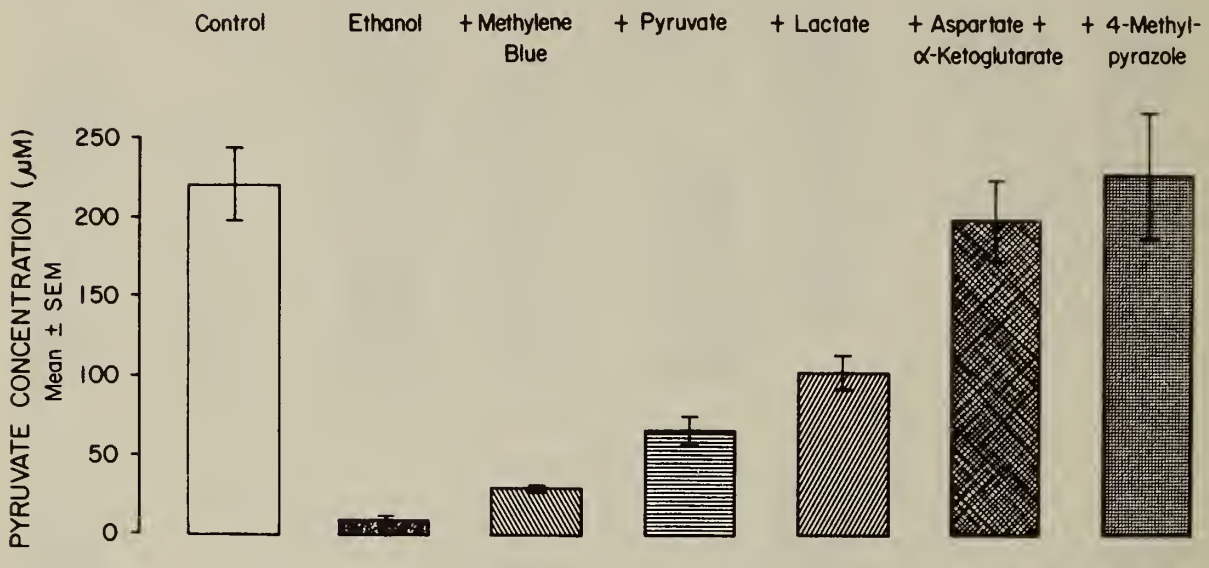
Note: Removal of reducing equivalents from the cytosol either by methylene blue (MB, an artificial scavenger of reducing equivalents) or by facilitation of their transport into the mitochondria (with substrates for the malic-aspartic acid shuttle: aspartate [ASP] and α-ketoglutarate [αK] markedly increased the accumulation of acetaldehyde. Despite the increase in acetaldehyde concentration, the inhibition of protein synthesis was prevented.

Figure 10. Relationship Between Alterations of Cytosolic Redox State and the Ethanol-Induced Inhibition of Protein Synthesis in Hepatocytes



Note: Addition of pyruvate attenuates the shift in cytosolic redox state (as judged from lactate/pyruvate ratio) and prevents the ethanol-induced inhibition of protein synthesis. However, the addition of lactate, which exaggerates the redox shift, also prevents the inhibition of protein synthesis.

Figure 11. Changes in Pyruvate Concentration Produced by Ethanol and by Agents That Prevent the Ethanol-Induced Inhibition of Protein Synthesis in Hepatocytes Isolated From Fed Rats



Note: The inhibition of protein synthesis produced by ethanol is associated with depletion of pyruvate. Agents that prevent the inhibition of protein synthesis spare pyruvate from depletion.

Table 1. Changes in Ethanol Metabolism (With and Without 4-Methylpyrazole) Induced by Chronic Alcohol Consumption in the Rat

	I.V. Ethanol (1.8 g/kg)		I.V. Ethanol (1.4 g/kg) + 4-Methylprazole (14 mg/kg)	
	Ethanol-Fed Rats	Pair-Fed Controls	Ethanol-Fed Rats	Pair-Fed Controls
Ethanol oxidation rate (nmol/kg/h)	9.30 ± 0.96	6.30 ± 0.66	2.24 ± 0.05	1.90 ± 0.05
	$p<0.01$		$p<0.01$	
Liver ethanol concentration at 90 min (μmol/g)	26.6 ± 1.9	34.4 ± 2.4	29.9 ± 1.8	30.5 ± 0.9
	$p<0.01$		N.S.	
Liver acetaldehyde concentration at 90 min (nmol/g)	34.9 ± 2.7	27.5 ± 2.9	19.9 ± 1.3	14.7 ± 0.8
	$p<0.05$		$p<0.02$	

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Transfer RNA Availability and Protein Synthesis

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Abstract

Gene expression in prokaryotes is controlled mainly at the level of transcription. In eukaryotes, which have more stable messenger RNAs, regulation may also occur at the level of translation. One possible factor is the availability of transfer RNAs. Variability in the amounts of specific tRNAs and the degree of aminoacylation has been noted in microorganisms and in plant and animal cells under various physiological conditions and in response, for example, to carcinogens, virus infection, and hormones. Intracellular compartmentalization of protein synthesis and tRNA aminoacylation has been documented. Alterations in isoaccepting tRNA patterns may occur through changes in the rate of synthesis or pre-tRNA maturation or degradation. Furthermore, changes in conformation and modification of preexisting tRNAs may have an effect on their use in protein synthesis. The hypothesis of functional adaptation of tRNA population for protein synthesis (Garel 1974) is discussed in light of recent developments.

Introduction

Transfer RNA plays a central role in protein synthesis. It translates the genetic message and is the molecule by which both the amino acid and the growing peptide chain are attached to the ribosome. Translation proceeds in two stages. The first is aminoacylation, by which an amino acid is attached to the tRNA specific for that amino acid. The second is the process of elongation, which first involves the codon-dependent binding of aminoacyl-tRNA to the ribosomal A site in which tRNA anticodon-codon recognition is

used to select the specific aminoacyl-tRNA. After that follows the formation of a peptide bond and the translocation of the growing peptide chain from A to P site on the ribosome. Since only the tRNA portion of aminoacyl-tRNA is involved in the binding to the ribosome, aminoacylation must be at least as specific as codon-anticodon pairing to insure the fidelity of protein synthesis. There is only one synthetase in the prokaryotic cell for each amino acid, and the same is probably true in the eukaryotic cell, although it is less certain because additional synthetases are coded by the organelle DNA (Schimmel and Söll 1979).

There are typically 40 to 60 tRNA species with different sequences in the cytoplasm of the eukaryotic cell. Thus, there are several tRNAs called (true) isoacceptors for each amino acid. In addition, there are 20 to 25 tRNAs in organelles such as mitochondria and chloroplasts. They are used in organelle protein synthesis (Barrell et al. 1980; Bonitz et al. 1980; Heckman et al. 1980). Isoacceptors differ in the sequence of their anticodon, since their function is to translate the several degenerate codons for each amino acid. It can be shown that, with the rules of wobble pairing, a minimum of 31 types of tRNA are needed to read the standard genetic code. However, only 23 to 24 tRNA genes exist in mitochondria, and the codon-anticodon recognition rules in mitochondrial protein synthesis are somewhat different from those used in the cytoplasmic protein synthesis (Barrell et al. 1980; Bonitz et al. 1980; Heckman et al. 1980). Typically, mitochondrial tRNAs contain an unmodified U in the first position of the anticodon, which allows a less restrictive codon-anticodon pairing (Heckman et al. 1980).

Transfer RNAs also differ in a number of other sequence positions, although isoacceptors generally show partial sequence homology with each other. Each of the more than 200 tRNA molecules whose primary structure is now known contains from 73 to 93 nucleotides with a content of modified nucleotides as high as 20 percent in tRNAs of higher organisms (Sprinzl et al. 1980). There are also pseudo-isoacceptors, tRNAs which differ from each other by only a few nucleotides (Raba et al. 1979). They are probably a result of tRNA gene duplication and subsequent mutation.

Transfer RNA Synthesis and Maturation

In bacteria, as well as in the cells of animals and plants, there are multiple copies of tRNA genes, amounting to about 60 in *E. coli* (Altman 1978). In eukaryotes, tRNA genes are amplified several hundred times. The multiplicity of tRNA genes may be needed to satisfy a quantitative requirement for protein synthesis on many ribosomes during growth and development. tRNA genes are not associated with the nucleolar organizer, as are the rRNA genes, but are dispersed among many chromosomes (Altman 1978; Beckman et al. 1979). In *E. coli*, certain tRNA genes are located within and in close proximity to the 6 to 10 ribosomal RNA cistrons (Altman 1978). A transcript is made of the whole cistron, which is then cleaved. It is thus clear that in bacteria the synthesis of a number of tRNA species is coordinately controlled with the synthesis of rRNA. Certain tRNA genes in mitochondria are also closely associated with the rRNA genes and are probably transcribed together with the rRNA genes (Eperon et al. 1980). In nuclei, however, multiple tRNA gene copies appear in tandem, as in *Xenopus*, while in other cases, as in yeast and *Drosophila*, the genes are dispersed (Beckmann et al. 1979; Hovemann et al. 1980).

Eukaryotic tRNA precursors are typically monomeric, with a small number of extra nucleotides at each end (Altman 1978). Some, but not all, eukaryotic precursor tRNAs contain an intervening sequence of 13 to 34 nucleotides inserted close to the 3′ side of the anticodon (Mao et al. 1980; Ogden et al. 1979). The precursor tRNAs lack the common CCA end, which is added later by a specific tRNA nucleotidyl transferase. Processing and conversion to minor bases as well as splicing of the intervening sequence occur in the nucleus, but final maturation, which includes additional base modifications, takes place in the cytoplasm (Altman 1978; Melton et al. 1980). The minor bases are all made by modification of the original nucleotide in the precursor polynucleotide chain except for the base Q, which is inserted by base exchange with G. A major role for the modified nucleotides in tRNA appears to be in codon-anticodon recognition, although other roles for the minor bases have also been suggested (Nishimura 1979).

The tRNA Cycle

Each time tRNA is involved in amino acid incorporation, it is cycled through several stages. These include free tRNA without an esterified amino acid, tRNA or aminoacyl-tRNA attached to its specific synthetase, free aminoacyl-tRNA, aminoacyl-tRNA attached to the elongation factor (EF-1α•GTP) or to its codon in the ribosomal A site, and peptidyl-tRNA in the ribosomal P site. After the nascent peptide chain in the tRNA is transferred to the aminoacyl-tRNA on the next codon, the tRNA is released and the cycle begins again. The rapidity of tRNA cycling depends on the abundance of a specific tRNA species in the cell, on the frequency with which it participates in protein synthesis, its rate of aminoacylation, its affinity for EF-1 and its codon in the A site, and the duration of its ribosomal attachment. The affinity of synthetases for tRNA is very high ($\cong 10^8\ M^{-1}$), and the intracellular concentrations of tRNA are typically of the order of magnitude of the Km constants that have been calculated for tRNA in aminoacylation, suggesting that there is generally very little free tRNA in the cell (Schimell and Söll 1979; Smith 1975). Attachment of aminoacyl-tRNA to the synthetase and EF-1 also helps to protect the labile aminoacyl-tRNA bond from premature hydrolysis.

Other tRNA Functions

In addition to the functions of tRNA in ribosomal protein synthesis, certain tRNA species are involved in other less well understood reactions. Among them is the ability of some eukaryotic tRNAs to serve as primers for the reverse transcriptase of RNA tumor viruses (Clark 1979). Another function of aminoacyl-tRNA is as a donor of activated amino acid for N-terminal addition of preexisting proteins in both prokaryotes and eukaryotes and for bacterial cell wall peptidoglycan synthesis (Clark 1979). Compartmentation of tRNAs may restrict their use in protein synthesis. Organelle tRNAs are one such example. At least in yeast mitochondria, no cytoplasmic tRNAs are needed for protein synthesis (Dirheimer et al. 1979). In previtellogenic oocytes of amphibians and teleosts, a bulk of tRNA is associated with 5S RNA and two proteins in a high-molecular-weight complex sedimenting at 42S (Picard et al. 1980). All kinds of tRNA are present in these

particles, and particle tRNA seems to be fully aminoacylated in vivo (Wegnez and Denis 1979).

Functional Adaptation of tRNA Population

The concentration of tRNA in the cell is not much higher than the concentration of ribosomes (calculated as monomers). There are more than 10 tRNAs per ribosome in *E. coli*, whereas in rabbit reticulocyte there are 4 to 5 tRNAs per ribosome (Smith 1975; Watson 1976). These estimations and the frequent finding of alterations in the tRNA population under various physiological and pathological conditions have led to proposals that tRNA may play a regulatory role in protein synthesis (Littauer and Inouye 1973).

Control of translation by the availability of tRNA may act both negatively and positively. Limiting amounts of certain tRNA species may delay translation at codons where they are required. On the other hand, enrichment of certain tRNA species, e.g., during cellular differentiation, may facilitate the synthesis of proteins whose translation would have been limiting previously, a process that Garel (1974) called "functional adaptation of tRNA population" and Smith (1975) called "tRNA specialization." Several observations now indicate that both tRNA specialization and tRNA availability can be determinants of the rate of protein synthesis.

The concept of tRNA-controlled translation was first proposed by Ames and Hartman (1963) to explain polarity in the synthesis of the enzymes of the histidine operon in *Salmonella*. Later, Itano (1965) suggested that the availability of certain aminoacylated tRNA species may limit hemoglobin synthesis in sickle cell heterozygotes. To test this hypothesis, Anderson and Gilbert (1969) added chromatographically purified fractions of reticulocyte tRNA to a cell-free system obtained from reticulocytes and were able to alter the ratio of the amount of α-globin synthesized to the amount of β-globin synthesized. The suggestion was made that the rate of translation of at least one globin mRNA was altered by changes in the availability of one or more tRNA species. It has also been shown in several in vitro systems that messenger tRNAs are better translated with homologous tRNAs than with heterologous tRNAs, thus providing evidence that variations in animal cell tRNA populations do have the potential for restricting the translation of certain classes of mRNA (Gerlinger et al. 1975; Sharma et al. 1976).

In cells committed to synthesis of one or only a few types of proteins, the tRNA population tends to correlate with the amino acid composition of the synthesized proteins. For example, such observations have been made in silk gland of *Bombyx mori* (fibroin synthesis), chick embryo tissues and rat granulation tissue (collagen synthesis), cell lines synthesizing specific immunoglobulins, avian liver stimulated by estrogens (vitellogenin synthesis), and rabbit reticulocytes or Friend leukemia cells stimulated by dimethylsulfoxide (hemoglobin synthesis).

Fibroin Synthesis

In the silk gland of the silkworm *Bombyx mori*, the codon distribution of fibroin mRNA is peculiar. Glycine is almost exclusively coded by GGU and GGA, and alanine is mainly coded by GCU. Together, glycine, alanine, and serine account for about 90 percent of the amino acids in fibroin. The corresponding glycine, alanine, and serine tRNA species predominate in the posterior silk gland, and the relative abundance of their isoacceptors is closely correlated to the codon frequency of fibroin mRNA (Chavancy et al. 1979; Garel 1974). Starvation and refeeding experiments have indicated that the rates of synthesis of different tRNAs vary widely according to the intracellular levels of each tRNA species, and the authors concluded that specific tRNA levels in this system are controlled at the transcriptional level or at the pre-tRNA maturation level (Fournier et al. 1976). This conclusion has been criticized, however, by Litt and Howell-Litt (1980), who have demonstrated that amino acid deprivation causes alterations in the rate of degradation of specific tRNA species.

Collagen Synthesis

Collagen is also an unusual protein because it contains 33 percent glycine and 22 percent proline plus hydroxyproline residues. The hydroxylation of certain proline and lysine residues occurs postribosomally. An increase in proline, glycine, and lysine acceptance of unfractionated tRNA derived from rat granulation tissue and from chick embryo collagen synthesizing tissues has been demonstrated (Christner and Rosenbloom 1976; Lanks and Weinstein 1970; Mäenpää and Ahonen 1972). In addition, it has been found that only one glycine isoacceptor (cognate to GGU and GGC) is increased

in amount and is preferentially used in polyribosomal glycine incorporation (Carpousis et al. 1977; Drabkin and Lukens 1978).

Immunoglobulin Synthesis

Marini and Mushinski (1979) and Mushinski et al. (1980) have recently analyzed the chromatographic profiles of aminoacyl-tRNAs for all 20 amino acids from 11 mouse plasmacytomas and from adult mouse liver and brain. The patterns of isoacceptors were compared statistically with the structure of the myeloma proteins being produced using so-called dissimilarity indices. Cluster analysis indicated that although myeloma protein is only one of many proteins being produced by the malignant cells, independently arising tumors have more similar profiles if the immunoglobulin light and heavy chains are very similar than if these chains are dissimilar. However, drastic changes in myeloma protein synthesis, such as loss of both heavy and light chain synthesis, did not result in increased dissimilarity of aminoacyl-tRNA profiles. Thus, these cells did not seem to adapt their tRNA population to a loss of specific protein synthesis.

Vitellogenin Synthesis

Vitellogenin is the yolk protein precursor found in the blood of laying oviparous vertebrates. Its synthesis in the liver can be induced also in immature and male animals by estrogens. In the ovary, vitellogenin is cleaved into lipovitellin (170,000) and two slightly different phosvitins (28,000 and 34,000). Vitellogenin contains about 3 percent phosphorus by weight, and most of the phosphorus is in the phosvitin moiety, which is composed of about 50 percent clustered serine residues, over 90 percent of which appear to be phosphorylated (see Linqvist and Mäenpää 1980). In estrogen-stimulated chicks and roosters, the hepatic synthesis of vitellogenin is accompanied by a 16 to 25 percent increase in the serine acceptance of hepatic tRNA (Klyde and Bernfield 1973; Mäenpää and Bernfield 1969). Of the four serine isoacceptors, $tRNA^{Ser}$ recognizing UCU, UCC, and UCA is relatively enriched in the tRNA preparation containing predominantly cytosolic tRNA, whereas the $tRNA^{Ser}$ species recognizing AGU and AGC is enriched in the membrane-bound ribosomes (Mäenpää and Bernfield 1975). No appreciable change in the relative rate of $tRNA^{Ser}$ synthesis was

found in double-labeling experiments during a period when the level of $tRNA^{Ser}$ continued to increase (Klyde and Bernfield 1973). Furthermore, the rates of synthesis of the two major $tRNA^{Ser}$ species were measured separately and found to be roughly similar (Kanerva and Mäenpää 1978). In recent experiments, we have found, however, that the degradation of the $tRNA^{Ser}$ species recognizing AGU and AGC is much slower during vitellogenin synthesis than in uninduced animals (Kanerva and Mäenpää unpublished). Together, these results suggest that the $tRNA^{Ser}$ isoacceptors adapt to vitellogenin synthesis by selective changes in $tRNA^{Ser}$ degradation in addition to a general increase in $tRNA^{Ser}$ transcription.

Hemoglobin Synthesis

Hemoglobin synthesis constitutes approximately 90 percent of the total protein synthesis in reticulocytes. Smith and his coworkers have studied extensively the specialization of reticulocyte tRNAs for hemoglobin synthesis (for a review, see Smith 1975). A strong positive correlation was observed when the amount of the amino acids in rabbit hemoglobin was compared with the relative abundance of tRNA for the different amino acids. There are two exceptions to this relationship: $tRNA^{Met}$ is abundant in reticulocytes compared to the amount of methionine in rabbit hemoglobin, even allowing for the labile methionine residue that is removed after initiation, and $tRNA^{Leu}$ is scarce compared to the amount of leucine in hemoglobin. The relative abundance of tRNA was also compared in rabbit liver and reticulocytes. $tRNA^{His}$ was found to be three times more abundant in reticulocytes than liver, and $tRNA^{Ile}$ was two times less abundant. Histidine is a relatively common amino acid in hemoglobin, and isoleucine is scarce. Recently, these authors also demonstrated that the relative abundance of $tRNA^{His}$ and $tRNA^{Ile}$ in early precursor cells of rabbit reticulocytes is not like that of reticulocytes but resembles that of liver (Smith et al. 1980). Thus, typical features of adapted tRNA content in reticulocytes are not seen in the red cell precursor cells, which do not synthesize hemoglobin.

Hatfield et al. (1979) have compared the chromatographic profiles of aminoacyl-tRNAs for all 20 amino acids from rabbit reticulocytes to those of rabbit liver by RPC-5 chromatography. The two tissues showed significant differences in the elution profiles of

most aminoacyl-tRNAs. The patterns of codon recognition of several purified aminoacyl-tRNA species indicated that some isoacceptors in rabbit liver, a tissue that is engaged in making a large number of proteins, were not observed in reticulocytes. Isoacceptors of glycine and leucine that recognized GGA and CUA, respectively, were observed in the tRNA from liver but not in the tRNA from reticulocytes. Furthermore, the distribution of codon-specific isoacceptors varied within the tRNA populations from a single tissue as well as between the tissues. Thus, in reticulocytes the most abundant leucine isoacceptor recognized CUG, whereas that which recognized UUG occurred in minor amounts. A serine isoacceptor recognizing UCG was scarce compared to the other serine isoacceptors in reticulocytes. Isoacceptors of glutamine, glutamic acid, and lysine, which recognized CAA, GAA, and AAA, were present in lower amounts in reticulocytes than the corresponding isoacceptors, which recognized CAG, GAG, and AAG. The elucidation of the rabbit α- and β-globin mRNA sequences now make possible a comparison of the abundances of codon-specific aminoacyl-tRNA species with the utilization of the corresponding codons in globin mRNAs. Rabbit globin mRNAs do not contain CAA, GGA, CUA, or UCG codons for glutamine, glycine, leucine, or serine. Isoacceptors corresponding to these codons occurred in reticulocytes in minor amounts compared to the other isoacceptors of the same amino acid. In addition, 30 of the 35 leucine residues in hemoglobin are coded by CUG and only one by UUG, and more of the lysine residues are coded by AAG than by AAA, and more of the glutamic acid residues are coded by GAG than by GAA.

Friend leukemia cells, which undergo erythroid-like differentiation after induction with dimethylsulfoxide, have also been studied in an effort to correlate the changes in isoaccepting tRNAs to hemoglobin synthesis. Lin and Agris (1980) and Lin et al. (1980) analyzed 64 isoacceptor species of tRNAs for 16 amino acids by RPC-5 chromatography. The relative amounts of $tRNA^{Phe}$, $tRNA^{Ile}$, and $tRNA^{Val}$ were maintained relatively constant during differentiation, whereas the relative abundance of some of the other isoacceptors for the other 13 amino acids changed significantly. In most cases, the predominant isoacceptors of uninduced cells were retained throughout differentiation. Exceptions were tRNAs for threonine, proline, and methionine. Some minor isoacceptors were not expressed at all times. In addition, the amount of Q-base-containing tRNA species decreased during the first 48 hours after the induction and then increased again, indicating that the level of

the Q-mopdification is correlated to the process of differentiation. Q-base, or its glycoslyated derivative Q*, occurs typically in the first position of the anticodon in tRNAs specific for asparagine, aspartic acid, histidine, and tyrosine and is thought to be involved in codon-anticodon interaction.

Aminoacylation Levels of tRNA

In bacteria, the study of some operons directing the biosynthetic pathways of amino acids (essential to humans), such as histidine, phenylalanine, tryptophan, threonine, and leucine, has revealed that, in addition to a control occurring at the initiation of transcription, which in some cases involves repressor molecules and various cofactors, there exists regulation of premature termination of transcription that takes into account the degree of aminoacylation of specific tRNA species (Crawford and Stauffer 1980). This attenuation mechanism is probably a means by which the bacterial cell can adapt to conditions of mild or severe amino acid deprivation. Another important regulatory mechanism in bacteria that involves unacylated tRNA is the regulation of accumulation of guanosine tetraphosphate (ppGpp) during the stringent control (Gallant 1979).

In cells of higher organisms, regulation based on the levels of aminoacylation of tRNA has been suggested in the expression of amino acid biosynthetic enzyme levels. Thus, in mutants of Chinese hamster ovary cells, asparagine synthetase activity is increased twofold to threefold when the cells containing the temperature-sensitive asparaginyl-tRNA synthetase are shifted to elevated temperatures at asparagine concentrations sufficient to fully repress enzyme activity at the permissive temperature. However, a specific and direct role of $tRNA^{Asn}$ in the regulation of asparagine synthetase levels seems to be ruled out by the finding that an elevated enzyme activity could also be correlated with decreased aminoacylation of other tRNAs such as $tRNA^{Leu}$, $tRNA^{Met}$, and $tRNA^{Lys}$ (Andrulis et al. 1979).

Friend leukemia cells have been shown to respond to deprivation of specific amino acids (histidine, leucine, tryptophan, or phenylalanine) by increasing their relative concentration of the cognate tRNA (Litt and Howell-Litt 1980; Litt and Weiser 1978; Weiser and Litt 1979). Studies with $tRNA^{Phe}$ have recently indicated that

deprivation of phenylalanine has no effect on the relative rate of synthesis of $tRNA^{Phe}$, but that it induces a decline in the relative rate of degradation of $tRNA^{Phe}$, which can also explain the increase in the relative abundance of $tRNA^{Phe}$ in these cells (Litt and Howell-Litt 1980).

Conclusions

Gene expression in prokaryotes is controlled mainly at the level of transcription. In eukaryotes with more stable mRNAs, regulation (positive or negative) may also occur at the level of translation. One possible regulatory factor is the availability of tRNA. The frequent finding that tRNA utilization and tRNA concentration are correlated indicates that tRNA has a role in regulating its own concentration in eukaryotic cells, although the mechanisms by which tRNA levels influence tRNA degradation in the cytoplasm and the transcription of specific tRNA genes in the nucleus remain unknown.

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Consequences of Maternal Alcoholism on Protein Metabolism in the Fetus and the Newborn*

Arun K. Rawat

Abstract

Metabolic consequences of short- and long-term ethanol consumption on protein metabolism in pregnant and lactating rats have been investigated in fetal and neonatal rat organs. The present study specifically examines the effects of maternal ethanol consumption (a) on fetal and neonatal cerebral, hepatic, and cardiac protein synthesis; (b) on proteolysis and the enzymes of protein synthesis and degradation in the fetal and newborn organs; and (c) on RNA and DNA contents of fetal and neonatal brains, livers, and hearts. Ethanol was given as 30 percent of total calories in isocaloric liquid Sustacal-ethanol/sucrose diet in long-term experiments; the animals were pair-fed. Prolonged maternal ethanol consumption during gestation or lactation resulted in a significant ($p < 0.001$) inhibition in the cerebral, hepatic, and cardiac rates of in vitro protein synthesis compared to the corresponding controls. Although the most active protein synthesis was observed in the newborn brains, the highest inhibition in protein synthesis was observed in fetal brains from ethanol-fed group. Both fetal and neonatal brains from ethanol-fed mothers showed a significant decrease in t-RNA formation compared to corresponding controls. In vivo studies showed that intravenously injected (^{14}C)leucine into hepatic proteins and hepatic tryptophan oxygenase activity were significantly lower in fetal, neonatal, and adult livers from ethanol-fed group. Pyrazole addition to adult whole liver

NOTE: Tables appear at end of paper.

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preparations partially prevented the effects of externally added ethanol on protein synthesis. In cardiac tissue, in vitro addition of ethanol did not affect cardiac protein synthesis in fetal, neonatal, and adult preparations. However, acetaldehyde addition resulted in an inhibition in neonatal and adult hearts. Acetaldehyde-medicated inhibition of cardiac protein synthesis was partially prevented by disulfiram. Cross-mixing experiments with their ribosomes and pH5-enzyme fractions from cerebral, hepatic, or cardiac preparations showed that in all three organs, the capacity of both fractions to synthesize protein was decreased by prolonged ethanol feeding. Although prolonged maternal ethanol consumption resulted in a significant decrease in the fetal and neonatal contents of RNA in all three organs, only the content of cerebral DNA showed a decrease. A demonstrable increase could not be observed in proteolytic activity as a result of maternal alcoholism in cerebral, hepatic, or cardiac tissues of the fetuses and the newborns.

Introduction

Recent studies have shown that both short- and long-term ethanol administration, either during gestation or lactation, result in a variety of alterations in the metabolic pathways of various organs of the developing fetus and the newborn. Some of these changes in lipid, carbohydrate, neurotransmitter, and drug metabolic pathways in the fetus and newborn have been reviewed recently (Rawat 1976*a*, 1980*a*, *b*, *c*). The clinical (Rosett 1976) and behavioral aspects (Abel 1980) have been reviewed elsewhere.

The present paper will review the studies done in our laboratory relating to the metabolic consequences of maternal alcoholism on protein synthesis in fetal and neonatal rat organs, specifically in the brain, liver, and heart.

In the brain, ethanol has been shown to produce prompt and profound psychopharmacological effects (Hawkins and Kalant 1972; Rawat 1975; Rix 1977; Sytinski 1980; Wallgren and Barry 1970). It is generally accepted that macromolecules such as proteins and nucleic acids play an important role in memory functions, learning, recall, retention, and even intelligence. Although dramatic effects of ethanol on the adult brain have been documented, such data on the developing brain have been scanty to date.

Mental retardation and neurological and behavioral abnormalities have been reported in the offspring of alcoholic mothers; however, the metabolic events leading to such anomalies have not been widely investigated. In the course of its development, the brain goes through several periods of rapid growth, during which it is extremely vulnerable to exogenous insults.

In the liver, it has been suggested that ethanol impairs the release of lipoproteins (Isselbacher and Greenberger 1964) and decreases the synthesis of albumin, with polysome disaggregation (Rothschild et al. 1975). Ethanol has been observed to decrease transferrin and albumin within hours after its oral administration, accompanied by an effect on hepatic endoplasmic reticulum (Jeejeebhoy et al. 1975; Nadkarni 1974).

In the heart, chronic alcoholism and/or malnutrition have been shown to be associated with cardiomyopathy in adults (Brigden et al. 1964; Evans 1961). Recent clinical observations have shown that offspring of alcoholic women are prone to congenital heart defects and abnormal growth (Jones et al. 1973; Lemoine et al. 1968). Although several congenital heart defects such as atrial septal defects, ventricular septal defects, and cardiac murmurs have been reported in infants born to alcoholic mothers (Jones et al. 1976), no metabolic studies are available to explain the possible mechanisms of such defects. Because the available evidence suggests that cardiac proteins are in a dynamic state of flux and that the protein synthesizing machinery shows very rapid responses to stress, the effects of short- and long-term ethanol consumption on fetal and newborn hearts during pregnancy and lactation will be examined.

The present article will mainly review the studies conducted in this laboratory on the metabolic consequences of maternal alcoholism on the protein synthesis of fetal and newborn organs. Although attempts to correlate the perinatal effects with adult effects will be made wherever necessary, a review of the effects of ethanol on adult organs is beyond the scope of this paper. Other researchers have reviewed the effects of ethanol on protein synthesis in the adult brain (Tewari and Noble 1979), liver (Jeejeebhoy et al. 1975), and heart (Rothschild et al. 1975).

Materials and Methods

Chemicals

ATP, GTP, and leucine were obtained from Sigma Chemical Co. (St. Louis, MO). (U^{14}C)leucine (270 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, MA). All chemicals used were of reagent grade. Enzymes and other biochemicals were obtained from Boehringer Mannheim Corp. (New York, NY).

Animals and Diets

Pregnant albino rats were obtained from Spartan Research (Haslett, MI). The animals for short-term experiments were maintained on Purina Rat Chow and water ad libitum. In experiments where the effects of acute ethanol administration were studied, ethanol (2 g/kg of body weight) was given by gastric intubation. For chronic ethanol treatment, several pairs of male and female rats were housed in separate cages. These paired animals were divided into control and ethanol-fed groups and were maintained on a Metrecal liquid diet upon the confirmation of pregnancy. For the neonatal study, newborns were suckled by mothers fed on either a Metrecal/sucrose or Metrecal/ethanol diet immediately after birth for a maximum of 10 days. The liquid diet consisted of 61 percent (v/v) commercial Metrecal (Mead Johnson Co., Evansville, IN; vanilla-flavored), 6 percent (v/v) ethanol or sucrose, and water. The daily dietary intake of liquid diet per experimental animal was measured. An equivalent amount of liquid diet containing sucrose was given to the control group. The details of dietary composition have been described previously (Rawat 1976*b*).

Studies With Fetal Brain, Liver, and Heart

To study the effects of chronic alcoholism, rats that were 18 to 21 days pregnant were used. The abdomens of the pregnant mothers were opened and the fetuses were dissected out while still connected to the maternal blood supply. The fetal organs were homogenized in 0.25 M sucrose/0.02M-Tris/HCI (pH 7.6)/10 mM magnesium acetate/40 mM NaCI/100 mM KCI/6 mM-2-mercaptoethanol. The homogenate was centrifuged at 0 to 4°C. Nuclear and mitochondrial fractions were prepared as described (Rawat and Kuriyama 1972). The supernatant obtained after sedimentation of the mitochondrial fraction was further centrifuged at 45,000 rev/min in a refrigerated Beckman L-2B 65 ultracentrifuge for 2 hours. The supernatant fraction was used for the preparation of pH5-enzymes as described by Rawat (1975). The resulting pellet was treated with sodium deoxycholate and was used for the preparation of purified ribosomes, as described by Munro et al. (1964).

Studies With Neonatal and Adult Brain, Liver, and Heart

The brains, livers, or hearts of newborn rats from 0 to 10 days after birth were used. The newborns came from either control or chronic ethanol-fed groups. In the control group, the newborn rats were suckled by Metrecal/sucrose-fed mothers, whereas in the alcohol group the neonatal animals were suckled by mothers fed on a Metrecal/ethanol diet. The adult animals used were 100 to 120 days old. Adult and neonatal animals were killed by cervical dislocation, and the organs were removed and ribosomes prepared as described above for fetal organs.

Incubation of Ribosomes With ($U^{14}C$)Leucine

The standard incubation mixture (1 ml) consisted of 0.25 M sucrose, 0.02 M Tris/HCI (pH 7.6), 0.11 M KCI, 0.04 M NaCI, 0.01 M magnesium acetate, 0.006 M GTP, 0.0040 M ATP, 0.1 ml of ribosomes, and 0.1 ml of pH5-enzyme protein. The final concentration of ($U^{14}C$)leucine was 2.5 μM (specific radioactivity 270 mCi/mmol, radiochemical purity 99 percent). Incubations were carried out in a metabolic shaker at 37°C for 30 minutes in an air atmosphere. The incorporation of ($U^{14}C$)leucine into protein was determined by the filter-paper-disc method (Mans and Novelli 1961). After adequate washing with 5 percent trichloroacetic acid and removal of amino-acid-charged tRNA and lipid material, the disc was counted for radioactivity in a Packard 385 liquid-scintillation counter. The formation of (^{14}C)leucyl transfer (t) RNA was carried out using the same incubation conditions described for (^{14}C)leucine incorporation into protein except for the deletion of ribosomes and GTP. In the isolation of (^{14}C)leucyl tRNA, the hot TCA extraction step was omitted. Incorporation into protein was calculated and expressed as cpm/mg of microsomal or pH5-enzyme protein. The total protein was determined colorimetrically (Lowry et al. 1951) with bovine serum albumin as protein standard.

Study of (^{14}C)Leucine Incorporation In Vivo

Trichloroacetic-acid-soluble and -insoluble radioactive samples were prepared as follows: control rats and rats that had been chronically fed ethanol were injected intravenously with ($U^{14}C$)leucine (3μCi). After 30 minutes the livers or hearts were

removed, weighed, and immediately homogenized in 5 ml of ice-cold 10 percent trichloroacetic acid. The homogenate was centrifuged at 15,000 rev/min in a refrigerated Beckman J-21 B centrifuge. The supernatant was extracted with 3x5 ml of diethyl ether to remove trichloroacetic acid. Portions of this supernatant were added directly to scintillation vials, and the radioactivity of the trichloroacetic-acid-soluble material was counted in a Packard Tri-Carb 385 liquid scintillation counter. The protein precipitate was washed with 3x10 ml of ice-cold 5 percent trichloroacetic acid and then incubated at 80°C for 20 minutes in 5 ml of 5 percent trichloroacetic acid to solubilize the nucleic acids. It was then washed with 2x10 ml of 5 percent trichloroacetic acid at 0°C, once with 10 ml of 70 percent (v/v) ethanol at 0°C, twice with 10 ml of 96 percent ethanol at room temperature, and finally once with 10 ml of ether at room temperature. The precipitate was transferred to filter-paper discs and counted for radiioactivity (Mans and Novelli 1961).

Incubation of Cardiac Homogenates

The effects of in vitro addition of ethanol (10 mM) or acetaldehyde (1 mM) were investigated on the cardiac whole homogenates. These tissue preparations were incubated in a Krebs-Henseleit bicarbonate buffer (pH 7.4) in an incubator shaker at 37°C. After 30 minutes of incubation, the reaction was terminated by the addition of perchloric acid (5 percent). Appropriate blanks were carried out, with perchloric acid added before the start of incubation. The incorporation of ($U^{14}C$)leucine into protein was determined by the filter-paper-disc method as described earlier for cardiac ribosomes.

Experiments with Cycloheximide, Pyrazole, and Disulfiram

In studies where the effect of cycloheximide addition on the rate of protein synthesis was investigated, homogenate was incubated with cycloheximide (2 mM) in a Krebs-Henseleit bicarbonate buffer. The incorporation of ($U^{14}C$)leucine into proteins was followed as described earlier in this section. Studies were also conducted with pyrazole (2 mM), an inhibitor of alcohol dehydrogenase, or disulfiram (2 mM), an inhibitor of aldehyde dehydrogenase. Details of determination of protein synthesis determination were similar to those described earlier in this section.

Determination of Total Proteins, RNA, and DNA Contents

Animals chronically fed ethanol (4 weeks), or animals from the corresponding isocalorically sucrose-fed group, were sacrificed by cervical dislocation. Samples of their organs were immediately removed for the determination of total protein (Lowry et al. 1951), RNA, and DNA (Rawat 1975) content.

Assay of Liver Enzymes

The activities of tryptophan oxygenase (EC 1.13.11.11) and tyrosine transaminase (EC 2.6.1.5) were assayed in whole homogenates of fetal, neonatal, and adult rat liver. The animals from both ethanol-fed and control groups were used.

Tryptophan Oxygenase Assay

The activity of tryptophan oxygenase was assayed by measuring the rate of kynurenine formation by the whole homogenates at pH 7.0. For routine analysis of tryptophan oxygenase activity, the whole homogenates were incubated with tryptophan in the presence of hemoglobin for 20 minutes. The amount of kynurenine formed during incubation was measured spectrophotometrically in the neutralized supernatants at 365 nm (Morland 1974). Tryptophan oxygenase activity is expressed in units (μmol of kynurenine formed per hour at 37°C per gram of wet liver tissue).

Tyrosine Transaminase Assay

The activity of hepatic tyrosine transaminase was measured by incubating the whole liver homogenate with a buffered medium (pH 7.4) containing phosphate buffer (0.2 M), pyridoxal phosphate (2.8 mM), 2-oxoglutaric acid (0.1 M), sucrose (0.25 M), and diethyldithiocarbamate (0.5 M). After a preincubation period of 3 minutes at 38°C, tyrosine (0.01 M) was added and the incubation was continued for another 10 minutes. The samples were filtered and the filtrate was used for the determination of color as described in detail previously (Rawat 1976*b*).

Determination of Proteolytic Activity

The effects of prolonged maternal alcoholism on the proteolytic activity of fetal and neonatal organs were determined with casein or hemoglobin as the substrates. The method described by Rick (1965) was employed.

Results

Protein Synthesis In Vitro

Brain Studies

The results of the effects of prolonged maternal ethanol consumption on the rates of (^{14}C)leucine incorporation by ribosomal and pH5-enzyme fractions from the brain, liver, and heart of the fetuses and neonates are described below.

It has been shown (Rawat 1975) that the chronic consumption of ethanol (6 percent v/v) for 2 weeks in liquid diet by the pregnant rats resulted in an inhibition in the rate of (^{14}C)leucine incorporation by the fetal cerebral ribosomes (table 1). The advantage of the present study over other studies in which ethanol is fed by drinking water or gastric intubation is that our animals were pair-fed on isocaloric sucrose and ethanol diets. This was to ascertain that while studying the effect of ethanol or brain protein synthesis, any nutritional imbalance was not due to ethanol administration. In an attempt to investigate that the transference of ethanol occurs through maternal milk, the effect of ethanol feeding to lactating mothers on the suckling neonates was investigated. As shown in table 1, the rate of (^{14}C)leucine incorporation by the cerebral ribosomes from neonatal animals suckling on alcohol-fed mothers showed a significant decrease ($p < 0.001$) compared with the corresponding neonates suckling on sucrose-fed mothers.

It was further observed that the chronic exposure of either pregnant mothers or lactating mothers to ethanol resulted in a significant effect on the pH5-enzyme fractions' capacity (reaction 2) to synthesize (^{14}C)leucine-tRNA. It suggests that chronic exposure to ethanol not only interferes with the capacity of cerebral ribosomes to synthesize proteins but also affects pH5-enzyme. Studies (table 2) with mixing various brain fractions of the fetuses from ethanol- and sucrose-fed mothers further support the possibili-

ty of a defect in both the ribosomes and the pH5 fractions of the alcohol group. The changes observed in the rate of protein synthesis as measured by the rate of (^{14}C)leucine incorporation in the fetuses and neonates from ethanol-fed group cannot be attributed to the metabolism of ethanol in the brain.

To study the in vitro response and sensitivity of cerebral ribosomal protein synthesis to ethanol at different developmental stages, the effect of ethanol addition was investigated. In vitro ethanol addition (50 mM) resulted in an inhibition of (^{14}C)leucine incorporation into cerebral protein from all fetal, neonatal, and adult ribosomes (table 3). Although the highest rates of protein synthesis were observed in the neonatal brains, the most pronounced inhibitory effect of ethanol addition was shown by the fetal ribosomes (table 3) and the lowest by the adult ribosomes. The inhibitory effect of ethanol on the rate of (^{14}C)leucine incorporation was found to be concentration dependent, regardless of age.

Liver Studies

As shown in table 4, ethanol 6 percent (v/v) consumption by pregnant rats or lactating mothers for 2 weeks resulted in an inhibition of the rate of (^{14}C)leucine incorporation into fetal and neonatal hepatic ribosomes compared to the pair-fed corresponding controls. The incorporation of (^{14}C)leucine into the hepatic ribosomes of adult animals was also significantly inhibited (table 4).

Furthermore, as shown in table 4, the prolonged and continuous exposure of either the pregnant mother or the lactating mother to ethanol resulted in a significant effect on the pH5-enzyme fractions of the fetal and neonatal livers to synthesize proteins. This suggests that such exposure to ethanol not only interferes with the capacity of fetal and neonatal hepatic ribosomes to synthesize proteins, but that it also affects the pH5-enzyme fraction. Although the possibility remains that administration of ethanol may result in a change in the precursor leucine pools, such changes were not observed.

As shown in table 5, the addition of ethanol in vitro (10 mM) resulted in a significant inhibition of the rate of (^{14}C)leucine incorporation into the adult liver. Preincubation of adult liver slices with pyrazole (2 mM) and subsequent addition of ethanol (10 mM) partially prevented the inhibitory effect of ethanol on the rate of (^{14}C)leucine incorporation into hepatic proteins. The addition of pyrazole alone to the adult liver slices did not significantly affect the rates of (^{14}C)leucine incorporation into hepatic proteins in vitro.

The inhibitory effect of ethanol on the rate of (^{14}C)leucine incorporation into hepatic proteins, and the partial prevention of this effect by the addition of pyrazole, was also observed in homogenates of adult livers. The addition of ethanol (10 mM) to adult liver ribosomes did not inhibit the rate of (^{14}C)leucine incorporation into ribosomal protein. Addition of pyrazole to the ribosomes from adult animals did not significantly inhibit the incorporation of (^{14}C)leucine into ribosomal proteins. Addition of ethanol to the homogenates of normal fetal livers did not significantly change the rates of (^{14}C)leucine incorporation into fetal hepatic proteins. Consequently, the preincubation of fetal liver homogenates with pyrazole and subsequent ethanol addition were also ineffective in changing the rates of (^{14}C)leucine incorporation into fetal liver proteins. Pyrazole alone did not significantly change the rate of (^{14}C)leucine incorporation into the proteins of fetal liver homogenates (table 5).

Studies that mixed hepatic ribosomal and pH5-enzyme fractions from the livers of fetuses from ethanol- or sucrose-fed mothers were conducted to determine the site of ethanol action. As shown in table 6, the lowest rates of (^{14}C)leucine incorporation were observed when the hepatic ribosomes and pH5-enzyme fractions from the ethanol-fed group were incubated together. Both mixing of pH5-enzyme fraction from the control group and ribosomes from the ethanol-fed group and mixing of pH5-enzyme fraction from the ethanol-fed group and ribosomes from the control group gave intermediate rates of protein synthesis (table 6).

Heart Studies

It is not surprising that ethanol consumption by the pregnant mother leads to a decreased rate of protein synthesis in the fetal heart (table 7), especially when one considers the free permeability of ethanol from maternal circulation to the developing fetus. The in utero environment, where the concentration of ethanol is roughly similar to that in the maternal blood, is obviously injurious to the normal development of the heart. The fact that ethanol impairs the capacity of both the ribosomes and pH5-enzyme fractions to synthesize proteins (table 8) further suggests that the developing heart of the newborn is quite susceptible to ethanol, which reaches the neonate from the maternal milk.

A lack of inhibitory effect of ethanol on the cardiac protein synthesis upon ethanol (10 mM) addition in vitro (table 9) suggests

that the effect of ethanol in the heart is at least not a consequence of its metabolism.

Protein Synthesis In Vivo

Liver Studies

Studies with (^{14}C)leucine administration intraveneously (Rawat 1976*b*) have shown that the prolonged consumption of ethanol by pregnant mothers also results in a significant inhibition of the overall capacity of the fetal liver to synthesize proteins. The rates of (^{14}C)leucine incorporation into fetal hepatic proteins were significantly lower in the ethanol-fed group compared to pair-fed controls. When (^{14}C)leucine was injected directly into the maternal circulation, it was observed that its incorporation into the trichloroacetic-acid-soluble fraction of fetal hepatic proteins from the ethanol-fed group was lower than that observed for the pair-fed control group (Rawat 1976*b*). Total incorporation of the label suggested that the rates of protein synthesis reached a peak in the livers from neonatal animals. The rates of protein synthesis in the fetal livers were lower than those observed in the neonatal and adult livers. The inhibitory effect of ethanol on protein synthesis was much more pronounced in the intact liver preparations than in the ribosomal preparations.

Heart Studies

The rates of (U^{14}C)leucine incorporation into tissue proteins under in vivo condition generally reflect the rates of protein synthesis. In other organs of body, such as the liver, the in vivo rates of (U^{14}C)leucine into the tissue proteins have been taken as the measure of protein synthesis. It was observed in the present study that the in vivo rates of (U^{14}C)leucine incorporation into cardiac total proteins and TCA-soluble fractions were significantly lower in the ethanol-fed animals than in the corresponding controls (Rawat 1979*a*).

Effects of Other Substances on Protein Synthesis

Brain Studies

Intraperitoneal injection of cycloheximide (500 mg/kg of body weight) to the neonates from ethanol- or pair-fed control groups resulted in about a 40 percent inhibition of brain protein synthesis in the ethanol-fed group and about a 60 percent inhibition in the pair-fed group (Rawat 1975). It has been previously observed that the in vitro addition of cycloheximide results in an inhibition in the incorporation of the labeled amino acid into ribosomal protein by the adult brain (Lamar 1972).

Liver Studies

Addition of cycloheximide in vitro resulted in an inhibition in the rates of (^{14}C)leucine incorporation by the hepatic ribosomes in newborn rats from both ethanol- and pair-fed groups (Rawat 1976*b*). This inhibition by cycloheximide was studied at 10 minute intervals up to a maximum of 30 minutes. Cycloheximide addition showed an inhibition at all the intervals tested. As described earlier, pyrazole (2 mM) addition to liver slices and liver homogenates partially prevented the inhibitory effect of ethanol on the hepatic protein synthesis (table 5). Addition of pyrazole alone did not have a significant effect on the rates of hepatic protein synthesis in vitro.

Cardiac Studies

The effects of the addition of acetaldehyde (1 mM) on the rates of cardiac protein synthesis by the whole homogenate preparations showed (table 10) that acetaldehyde had an even more pronounced inhibitory effect on the rates of protein synthesis than did ethanol (10 mM). The acetaldehyde-mediated inhibition of cardiac protein synthesis was at least partially prevented by preincubation with disulfiram (2 mM), an inhibitor of hepatic acetaldehyde dehydrogenase (table 10). Preincubation of cardiac homogenates with pyrazole (2 mM) did not have a significant effect on ethanol's effect on the heart (table 10). As mentioned earlier, addition of ethanol (10 mM) does not have a significant effect on cardiac protein synthesis (table 10). In heart homogenates, cycloheximide (2 mM) addition did have a significant inhibitory effect on protein synthesis.

Enzymes of Protein Metabolism

Protein Synthesizing Enzymes

Ethanol consumption by pregnant or lactating animals resulted in an inhibition of tryptophan oxygenase activity (EC 1.13.11.11.) in both fetal and neonatal liver (Rawat 1976*b*). Ethanol consumption also resulted in an inhibition of tryptophan oxygenase activity in the adult liver. This effect of ethanol was rather specific on the activity of tryptophan oxygenase, since maternal ethanol consumption did not result in a significant change in the activity of hepatic tyrosine aminotransferase (EC 2.6.1.5.).

Proteolytic Activity in the Liver

Chronic ethanol consumption by adult animals resulted in a decrease in hepatic proteolytic activity tested against hemoglobin (table 11). Proteolytic activity of liver against casein also decreased in the ethanol-fed group. Ethanol consumption by pregnant or lactating rats resulted in a small but significant decrease in the hepatic proteolytic activity in fetal and neonatal liver. Hepatic proteolytic activity showed a progressive increase from fetal to adult stage. The developmental changes in the hepatic proteolytic activity are expressed as percentages of that of the adult control.

Proteolytic Activity in the Heart

In an attempt to investigate whether the observed decrease in the protein content of the fetal and neonatal hearts from alcoholic mothers was a consequence of increased proteolysis, the effect of ethanol on proteolysis was investigated. As shown in table 12, prolonged maternal ethanol consumption during gestation and lactation did not result in a significant change in the proteolytic activity of the fetal and neonatal heart preparations.

Contents of RNA, DNA, and Proteins

Brain Studies

Because protein synthesis is under direct control of nucleic acids, the effect of chronic ethanol feeding to pregnant or lactating mothers has been investigated (Rawat 1975) on the fetal and neonatal cerebral content of RNA and DNA. Chronic ethanol

consumption by lactating mothers resulted in a significant decrease in total DNA content in the brains of both fetuses and neonates. The content of DNA-P in the brains of neonates suckling on sucrose-fed mothers was 96 ± 6 μg/g wet weight of brain, and in the ethanol-fed group it was 55 ± 4 μg/g wet weight of brain. The content of RNA-P in the sucrose-fed group was 100 ± 8 μg/g wet weight of brain, and it decreased to 78 ± 6 μg/g wet weight of brain in the ethanol-fed group. The water content of the brain, when expressed on the basis of percent wet weight of brain, did not show a significant difference between the neonates from control and ethanol groups. The percent water content of the control brains was 90 ± 0.3 and of the ethanol group was 92.3 ± 0.4. Therefore, the changes in the cerebral RNA and DNA contents after exposure to chronic ethanol treatment could not be accounted for by cerebral edema. A decrease in the content of these nucleic acids was also observed when the values were expressed on the basis of protein concentration. The protein content of the brain from the control group was 9.2 ± 0.12 percent, whereas the protein content of the ethanol group was 8.9 ± 0.5 percent. The values are from 10 animals in these groups and are expressed as mean ± SEM on the basis of wet weight of brain. Pups suckling on ethanol-fed dams showed a significant decrease in brain weight compared to the corresponding pair-fed controls. The average brain weights of the ethanol and control neonates were 368 ± 20 and 400 ± 40 mg, respectively. The observations are from 20 neonates in each group.

Liver Studies

As shown in table 13, maternal ethanol consumption for 2 weeks resulted in a significant decrease in the fetal hepatic content of total RNA as measured by RNA phosphorus content. Maternal ethanol consumption did not result in a significant change in the fetal hepatic content of total DNA. However, maternal ethanol consumption resulted in a significant decrease in the fetal hepatic RNA/DNA ratio. Ethanol feeding to the pregnant rats resulted in a decrease in total protein in the fetal liver; however, the RNA/protein ratio was not significantly affected by this treatment. Decreased RNA, RNA/DNA ratio, and liver protein contents in adult livers from ethanol-intoxicated rats have been previously reported (Albertini et al. 1970). A decrease in ribosomal protein content was also observed in fetal livers from ethanol-fed mothers compared to the corresponding pair-fed controls (table 14).

Cardiac Studies

Effects of prolonged ethanol consumption on the total proteins, RNA, and DNA in fetal and neonatal hearts are shown in table 14. Although ethanol consumption by pregnant or lactating rats resulted in a significant decrease in total proteins and RNA contents in the fetal and neonatal hearts, cardiac DNA content in either the fetal or neonatal rats did not show a significant change upon exposure to ethanol. Decreased cardiac total RNA and unchanged total DNA in ethanol-exposed neonates also have been reported by other laboratories (Henderson and Schenker 1977).

Discussion

Effects of prolonged ethanol administration on the rates of protein synthesis in adult liver (Morland 1974), brain (Kuriyama et al. 1971; Lamar 1972; Noble and Tewari 1973), pancreas (Tsuzuki et al. 1965), and heart (Rothschild et al. 1972) have been observed. However, no information is so far available in the literature about the effects of ethanol on protein synthesis in the developing organs. In 1975, our laboratory reported that prolonged maternal ethanol consumption by pregnant or lactating rats resulted in a significant inhibition in the rate of (^{14}C)leucine incorporation into fetal and neonatal cerebral ribosomes (Rawat 1975). Subsequent studies from this laboratory showed that such inhibitory effects of maternal ethanol consumption were also observed in the fetal and neonatal livers (Rawat 1976*b*) and hearts (Rawat 1979*b*). The inhibitory effect of ethanol on (^{14}C)leucine incorporation into fetal and neonatal ribosomes was observed on the basis of both ribosomal protein and pH5-enzyme proteins in all the organs tested. The results suggest that maternal ethanol consumption not only interferes with the capacity of fetal ribosomes to synthesize proteins but also affects pH5-enzymes.

Brain protein metabolism in adult animals after both acute and chronic ethanol administration has been studied by several investigators. In acute studies, ethanol either has been administered intraperitoneally, as in the study reported by Kuriyama et al. (1971) in which ethanol (4 g/kg) was injected into Swiss albino mice and a decrease in the in vitro incorporation of (^{14}C)leucine into proteins by a ribosomal system was observed, or has been administered intragastrically. In one such study, Renis et al. (1975) gave

acute ethanol (5 g/kg) to rats by stomach tube and found that (^{3}H)leucine incorporation into brain microsomal fraction was decreased while the mitochondrial fraction remained unaffected.

In chronic ethanol administration studies on brain protein synthesis, ethanol has been administered in the drinking water either as 10 percent aqueous solution, as in the studies by Noble and Tewari (1973), or as 15 percent aqueous solution, as in the studies of Järlstedt (1972).

Isocaloric pair-feeding of liquid diets has been the other mode of ethanol administration, as in the studies reported by Friedhoff and Miller (1973), Morland and Sjetnan (1976), and Rawat (1975). Ethanol administration in aqueous solution in our hands has not been satisfactory. The major problem has been that the ethanol-drinking group does not like the taste of ethanol aqueous solution, and it gives up drinking initially, whereas the control group keeps up its normal water intake. This leads to variability in the intake of ethanol and consequently to caloric imbalance.

Although several studies are available on the effects of ethanol on protein metabolism in the brains of adult animals, few studies have been done on fetal and neonatal effects (Rawat 1980*c*). The first study in this area, which was reported from our laboratory in 1975, showed that maternal ethanol consumption during gestation for 2 weeks resulted in about a 30 percent decrease in the incorporation of (^{14}C)leucine into protein of fetal cerebral ribosomes (table 1). It was further observed that the incorporation of (^{14}C)leucine into protein of cerebral ribosomes of newborn rats, suckling on ethanol-fed mothers for 4 days after birth, was 60 percent lower compared to the corresponding pair-fed control group (Rawat 1975). A subsequent study by another laboratory (Henderson and Schenker 1977) has confirmed these observations in the rat offspring by reporting a decrease in brain RNA and proteins.

The studies on the brain polysomes are conducted on both the free and the membrane-bound polysome population. However, in brain preparation, because there is a greater population of free ribosomes, the data most reflect the studies on free ribosomes. In the liver, a greater polysome population is membrane-bound and associated to the rough endoplasmic reticulum (RER). In the hepatic tissue, therefore, there is a greater possibility that the suggested role of ethanol (Rothschild et al. 1975) to induce polysome disaggregation of the membrane-bound preparations might play a greater role.

The chronic exposure of either pregnant or lactating mothers to ethanol resulted in a significant decrease in the capacity of the pH5-enzyme fractions (reaction 2) to synthesize (^{14}C)leucyl t-RNA. It suggests that chronic exposure to ethanol not only interferes with the capacity of cerebral ribosomes to synthesize proteins but also affects pH5-enzyme. Studies (table 2) on mixing various brain fractions of the fetuses from ethanol- and sucrose-fed mothers further support the possibility of a defect in both the ribosomes and pH5-enzyme fractions of the alcohol group. The changes observed in the rate of protein synthesis as measured by the rate of (^{14}C)leucine incorporation in the fetuses and neonates from ethanol-fed group cannot be attributed to the metabolism of ethanol in the brain. It is suggested that at least some of the observed changes are due to the effect of ethanol on aminoacylation process as a consequence of its effect on aminoacyl-t-RNA synthetases. The decrease in the content of RNA following chronic ethanol exposure (Rawat 1975) may be due to alterations in the processing of RNA in the brain. It is important to remember at this point that, in addition to its effect on aminoacyl-t-RNA synthetase, ethanol may also influence other factors in vivo that influence protein synthesis. In fact, both brain and liver ethanol administration have been shown to significantly alter the adenylate charge. Chronic ethanol administration in mice brain has been shown to decrease the levels of adenosine triphosphate (ATP) with a consequent increase in adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Rawat et al. 1973). Brain creatine phosphate levels were also found to decrease in this study upon chronic ethanol administration. In the liver, even acute administration of ethanol results in a lowering of ATP levels (Rawat 1968). Although there is no appreciable permeability barrier for ethanol from maternal circulation to the fetus via the placenta, it is possible that ethanol may selectively inhibit the transport of certain nutrients and precursors of protein synthesis from the mother to the fetus under the in vivo conditions. Since ethanol has been shown to alter the metabolism of gamma-aminobutyric acid (GABA) and its precursor glutamate in the brain (Rawat 1974; Rawat et al. 1973) and the metabolism of glutamate in the liver (Rawat 1968), the possibility that such changes in neurotransmitter metabolism may influence protein synthesis should also be considered.

In an attempt to investigate if the inhibitory effect of maternal ethanol consumption was also exerted on the developing fetus in situ, the experiments in vivo were designed. It was observed that

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ethanol consumption by pregnant rats was also inhibitory to the incorporation of (^{14}C)leucine into trichloroacetic-acid-precipitable and trichloroacetic-acid-nonprecipitable ninhydrin-positive material of fetal liver (Rawat 1976*b*) and heart (Rawat 1979*a*).

Although ethanol addition to adult rat liver slices has been observed to inhibit the incorporation of (^{14}C)leucine into hepatic proteins (Perin et al. 1974), the addition of ethanol in vitro to fetal liver slices or fetal hepatic ribosomes was ineffective in altering the rates of protein synthesis. The inhibitory effect of externally added ethanol on the rate of protein synthesis in the adult rat liver slices and the absence of this effect on the fetal liver slices suggests that at least part of the hepatic effect is mediated through the changes in hepatic NADH/NAD+ ratio through alcohol dehydrogenase. It has been shown that in the early stages of fetal development, the activity of alcohol dehydrogenase in the liver is either absent or very low (Räihä et al. 1967; Rawat 1976*c*).

The observation that in liver slices or liver homogenates the inhibitory effect of ethanol on the rate of protein synthesis, at least in part, is a consequence of ethanol-mediated changes in hepatic NADH/NAD+ ratio is further documented by the experiments with pyrazole (2 mM) in adult rat liver slices or homogenates; pyrazole was able to relieve, at least in part, the inhibitory effect of ethanol on protein synthesis. However, in the fetal liver slices, where the activity of alcohol dehydrogenase is either very low or absent, pyrazole was ineffective in relieving the ethanol-mediated inhibition. A lack of effect of pyrazole has also been observed in the guinea pig kidney slices, a tissue where alcohol dehydrogenase is not present to any significant extent (Rawat 1972). With the development of hepatic alcohol dehydrogenase, the effectiveness of pyrazole in relieving the ethanol-mediated inhibition of protein synthesis also increases, as observed in the adult liver (table 5).

Since the addition of ethanol in vitro to adult ribosomes does not significantly affect the rates of (^{14}C)leucine incorporation into protein (table 5), it becomes logical to suggest that the inhibitory effect of ethanol is exerted through two different mechanisms. The first mechanism, which is alcohol dehydrogenase dependent, operates in the whole liver and is sensitive even to the addition of pyrazole. The second mechanism, which is not dependent on alcohol dehydrogenase, operates in the ribosomal and pH5-enzyme fractions and is affected only on continuous and prolonged exposure to ethanol and is not sensitive to the addition of pyrazole.

It has been suggested that the effect of prolonged ethanol consumption on hepatic protein synthesis in adult mice may be a consequence of its effect on the concentrations of corticosteroids (Kuriyama et al. 1971). The effect of prolonged ethanol consumption by pregnant or lactating rats was therefore investigated (Rawat 1975*b*) on the activities of two hepatic enzymes, tryptophan oxygenase and tyrosine aminotransferase, both of which are induced by corticosteroids (Knox and Auerbach 1955). These two enzymes in liver have a very rapid turnover (Feigelson et al. 1959; Lin and Knox 1958), and their activities can therefore be easily influenced by changes in enzyme synthesis or breakdown. The developmental pattern of hepatic tryptophan oxygenase showed that during early stages of development, maternal ethanol consumption did not significantly affect the activity of tryptophan oxygenase (Rawat 1976*b*). However, the activity of tryptophan oxygenase decreased in the livers of newborn rats suckled by ethanol-fed mothers. Adult animals feeding on an ethanol diet showed a significant decrease in the activity compared to corresponding controls. A decrease in the adult hepatic tryptophan oxygenase activity has been observed (Morland 1974) in perfused rat liver preparations from chronically ethanol-fed animals. Although hepatic activity of tyrosine aminotransferase showed a steady linear increase with age, it was not significantly affected at any stage of development by ethanol (Rawat 1976*b*). These observations suggested that effect of ethanol on the activity of tryptophan oxygenase, tyrosine aminotransferase, or the rate of protein synthesis was not an indirect effect of ethanol exerted through corticosteroids. The latter have been shown to increase transiently in the plasma after ethanol administration (Ellis 1966).

A small but significant decrease in the contents of total hepatic protein in fetal, neonatal, and adult livers from the ethanol-fed group led us to investigate the effect of ethanol consumption on the proteolytic activity in liver. Observations in table 11, however, suggested that the effect of ethanol on hepatic protein content was not a consequence of increased protein breakdown, since the proteolytic activity in the fetal, neonatal, and adult livers did not increase in the ethanol-fed group. It can be assumed that the effect of ethanol on hepatic protein content is a consequence of decreased protein synthesis or increased leakage from the liver of the newly synthesized proteins. The inhibitory effect of ethanol on the rates of protein synthesis observed in the present study may be of central importance in the pathogenesis of alcoholic liver cirrhosis and

associated abnormal morphology of the liver frequently observed in chronic alcoholics.

Although several congenital heart defects such as atrial septal defects, ventricular septal defects, and cardiac murmurs have been reported in the infants born to human alcoholic mothers (Jones et al. 1973), no metabolic studies are available to offer the possible mechanisms of such defects. Chronic alcoholism has been shown to be associated with cardiomyopathy in human adults; however, not much information is available regarding the possibility of biochemical changes in the heart muscle of the offspring of alcoholic mothers. Earlier studies (Rawat 1979*a*) from this laboratory have shown that despite adequate nutrition, prolonged ethanol consumption results in protein synthesis inhibition in the adult heart. It was further observed that prolonged maternal ethanol consumption results in an inhibition in fetal and neonatal cardiac protein synthesis (Rawat 1979*b*).

Preliminary observations indicated that ethanol consumption during pregnancy or during periods of lactation resulted in a decrease in the total protein and RNA contents (table 14) in the heart. This led us to further investigate the rates of protein synthesis in the hearts of the alcoholic offspring. It is evident from the in vivo and in vitro experiments that prolonged ethanol consumption by the mother results in an inhibition in the rates of cardiac protein synthesis in both fetus and neonate. It is not surprising that ethanol consumption by the pregnant mother leads to the decreased rate of protein synthesis in the fetal heart, especially when one considers the lack of a permeability barrier of ethanol from maternal circulation to the developing fetus. The in utero environment, where the ethanol concentration roughly equals the maternal blood, is obviously injurious to the normal development of the heart. The fact that ethanol impairs the capacity of both the ribosomes and pH5-enzyme fractions to synthesize proteins in the neonatal hearts further suggests that the developing heart of the newborn is quite susceptible to ethanol, which reaches the neonate from the maternal milk.

A lack of inhibitory effect of ethanol on cardiac protein synthesis upon ethanol (10 mM) addition in vitro (table 10) suggests that the effect of ethanol in the heart is at least not a consequence of its metabolism. In perfused adult rat heart preparation, it has been observed (Rothschild et al. 1975) that acute administration of large doses of ethanol (250 mg/100 ml) were ineffective in altering the rates of cardiac protein synthesis. It is also clear from protein

degradation studies that the effect of ethanol on cardiac protein synthesis is not a consequence of increased cardiac proteolytic activity (table 12). However, since the addition of acetaldehyde (2 mM) is capable of inhibiting the rates of protein synthesis in vitro (Rawat 1979*b*), it is logical to suggest that acetaldehyde has a toxic effect on the heart protein synthesis. Acetaldehyde has been observed in the past to result in certain other metabolic and physiological derangements, such as cardiac acceleration and aortic hypertension in dogs (Chidsey et al. 1966). Cardiac anomalies, including septal defects and cardiac murmurs, have been observed in children born to chronically alcoholic mothers, and it is possible that at least some of the injurious effects of ethanol on cardiac tissue may be exerted by acetaldehyde through its protein-synthesis-inhibiting effect and its norepinephrine-depleting effect on the heart.

In conclusion, the data presented here demonstrate that prolonged maternal ethanol consumption leads to the inhibition of protein synthesis in the brain, liver, and heart of fetuses and suckling newborns on ethanol or isocaloric pair-fed mothers. In all three organs, the inhibitory effect of ethanol on protein synthesis is exerted at both the ribosomal and pH5-enzyme fraction levels. A decrease in the (^{14}C)leucyl-t-RNA formation in all three organs suggests that at least some of the effect of chronic maternal ethanol consumption on protein synthesis in fetal and neonatal organs is exerted at the aminoacyl-t-RNA synthetase level. In addition, ethanol, by virtue of altering the adenylate charge in the brain and liver, may also lead to an inhibition of protein synthesis. The ontogenesis of protein metabolism as influenced by maternal alcoholism has been studied here by using experimental techniques that minimize the influence of factors such as imbalanced nutritional intake and postmortem changes such as anoxia, hypoxia, and hypothermia. The effect of ethanol on protein synthesis is not exerted through the direct metabolism of ethanol except in the liver, where ethanol increases the hepatic NADH/NAD ratio, and the inhibitory effect of ethanol can be partially prevented by pyrazole.

It is possible that some of the observed anomalies in the fetal alcohol syndrome (FAS) may be at least in part attributable to the protein synthesis inhibition. It is generally believed that the macromolecules such as proteins and nucleic acids play an important role in memory functions, learning, recall, retention, and even intelligence. The observed inhibition of protein synthesis in the

brain may be linked to the observed intellectual deficit and mental retardation observed in FAS. Since proteins form the basic structural units of tissues such as heart muscle, it is possible that some of the cardiac structural anomalies observed in FAS, such as cardiac septal defects, may be attributable to the inhibitory effect of ethanol on the cardiac protein synthesis.

Table 1. Effect on Maternal Ethanol Consumption on (^{14}C)Leucine Incorporation in Fetal and Neonatal Brain Ribosomes and pH5-Enzyme Fraction

Treatment	(^{14}C)Leucine Incorporation (pmol/min/mg pH5 x ribosomal protein) I	(pmol/min/mg pH5-enzyme) II
Control fetus	5.76 ± 0.8 (8)	9.8 ± 1.0 (8)
Alcohol fetus	3.90 ± 0.7 (8)	4.8 ± 0.5 (8)
Control neonate	8.07 ± 1.0 (8)	15.9 ± 1.0 (8)
Alcohol neonate	3.40 ± 0.9 (8)	6.2 ± 0.5 (8)

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Note: Ribosomes were prepared from the brains of fetuses or neonates from alcohol-fed and control groups. Incorporation of (^{14}C)leucine into cerebral ribosomes in the presence of its corresponding pH5-enzyme is shown in the first column; incorporation without ribosomes, only in the presence of pH5-enzyme, is shown in the second column. The results are expressed as mean ± SEM, with the number of observations in parentheses.

Table 2. Effect of Mixing Various Brain Fractions of the Fetuses From Ethanol and Control Mothers

Source of Cerebral pH5-Enzyme	Source of Cerebral Ribosomes	
	Control Fetus	Ethanol Fetus
	(pmol of (^{14}C)leucine incorporated/mg protein/30 min)	
Control fetus	173.6 ± 15 (12)	123 ± 11 (12)
Ethanol fetus	110.0 ± 10 (12)	92 ± 8 (12)

SOURCE: Reproduced with permission from PJD Publications Limited, Westbury, N.Y., from Rawat, *Res Comm Chem Pathol Pharmacol*, 12(4):728, 1975. Copyright © 1975 by PJD Publications Ltd.

Note: Cerebral pH5-enzyme fractions and ribosomes were prepared from control and ethanol fetuses. The rates of (^{14}C)leucine incorporation by ribosomes from both control and ethanol-fed groups were studied in the presence of pH5-enzyme fractions from control fetuses and ethanol fetuses. The results are expressed as mean ± SEM, with the number of observations in parentheses.

Table 3. Effect of Ethanol Addition on the Rate of (^{14}C)Leucine Incorporation in Brain Ribosomal Protein

Brain Preparations (ribosomes and pH5-enzyme)	Additions	(^{14}C)Leucine Incorporation (pmol/min/mg pH5-enzyme x ribosomal protein)
Fetal (– 4 days)	– ethanol	5.80 ± 0.5 (8)
	+ethanol	2.03 ± 0.3 (8)
Neonatal (+ 4 days)	– ethanol	8.10 ± 1.0 (8)
	+ethanol	7.00 ± 1.0 (8)
Adult (+ 120 days)	– ethanol	6.30 ± 0.8 (8)
	+ethanol	6.00 ± 0.8 (8)

Note: Ribosomes and the corresponding pH5-enzymes were incubated in the presence or absence of ethanol. The details of the incubation procedure are given in the section on material and methods. Ethanol concentration was 50 mM. Results are expressed as mean ± SEM, with the number of observations in parentheses.

Table 4. Effect of Prolonged Maternal Ethanol Consumption on the Rate of (^{14}C)Leucine Incorporation Into Hepatic Ribosomal Protein in the Rat In Vitro

Age	Treatment	(^{14}C)Leucine Incorporation (pmol/30 min/mg protein) I	(^{14}C)Leucine Incorporation (pmol/30 min/mg pH5-enzyme) II
Fetal (–4 days)	Control	108 ± 6 (12)	205 ± 10 (12)
		$p<0.001$	$p<0.001$
	Ethanol	68 ± 6 (12)	115 ± 8 (12)
Neonatal (+10 days)	Control	152 ± 10 (10)	300 ± 15 (10)
		$p<0.01$	$p<0.01$
	Ethanol	100 ± 8 (10)	208 ± 10 (10)
Adult (+120 days)	Control	120 ± 10 (7)	245 ± 20 (7)
		$p<0.001$	$p<0.01$
	Ethanol	76 ± 5 (7)	160 ± 15 (7)

SOURCE: Rawat, *Biochem J,* 160:653-661, 1976, by permission. Copyright 1976 by The Biochemical Society, London.

Note: Effects of chronic ethanol consumption by pregnant, lactating, and adult animals on the rates of (^{14}C)leucine incorporation into ribosomal protein in the livers of fetuses, suckling newborns, and adult rats were studied. The ribosomes were prepared as described in the materials and methods section. The details of (^{14}C)leucine incorporation into ribosomal protein are given in the text. The results are expressed as means ± SEM, with the number of observations in parentheses.

Table 5. Effects of Addition of Ethanol and Pyrazole In Vitro on (^{14}C)Leucine Incorporation Into Hepatic Proteins in Adult and Fetal Rat Liver

Liver Preparation	Additions	Rates of (^{14}C)Leucine Incorporation (pmol/30 min/mg protein)	
		Adult	Fetal
Slices	Control	1250 ± 105 (6)	950 ± 86 (4)
		$p<0.01$	N.S.
	Ethanol (10 mM)	790 ± 80 (6)	900 ± 48 (4)
	Ethanol (10 mM)+pyrazole (2 mM)	1190 ± 130 (6)	938 ± 40 (4)
		N.S.	N.S.
	Pyrazole (2 mM)	1200 ± 145 (6)	943 ± 72 (4)
Homogenates	Control	805 ± 90 (6)	672 ± 50 (4)
		$p<0.01$	N.S.
	Ethanol (10 mM)	609 ± 60 (6)	585 ± 40 (4)
	Ethanol (10 mM)+pyrazole (2 mM)	785 ± 65 (6)	648 ± 60 (4)
		N.S.	N.S.
	Pyrazole (2 mM)	800 ± 85 (6)	680 ± 65 (4)

Table 5. Effects of Addition of Ethanol and Pyrazole In Vitro on (^{14}C)Leucine Incorporation Into Hepatic Proteins in Adult and Fetal Rat Liver (Cont.)

Liver Preparation	Additions	Rates of (^{14}C)Leucine Incorporation (pmol/30 min/mg protein)	
		Adult	Fetal
Ribosomes	Control	130 ± 20 (7)	108 ± 6 (12)
		N.S.	N.S.
	Ethanol (10 mM)	100 ± 10 (7)	108 ± 8 (12)
	Ethanol (10 mM)+pyrazole (2 mM)	120 ± 10 (7)	100 ± 6 (12)
		N.S.	N.S.
	Pyrazole (2 mM)	120 ± 20 (7)	110 ± 6 (12)

SOURCE: Rawat, *Biochem J,* 160:653-661, 1976, by permission. Copyright 1976 by The Biochemical Society, London.

Note: Effects of pyrazole addition on the inhibitory effect of ethanol on the rate of (^{14}C)leucine incorporation into adult or fetal liver slices, homogenates, or ribosomes were investigated. Hepatic preparations from normal adult or fetal livers were incubated at 37°C (pH 7.4), with the additions indicated in the table. The details of (^{14}C)leucine incorporation into hepatic preparations have been described in the text. The results are expressed as means ± SEM, with the number of experiments in parentheses. N.S. = Not significant.

Table 6. Effect of Mixing Hepatic Ribosomes and pH5-Enzyme Fractions of Fetuses From Ethanol-Fed and Control Groups on Protein Synthesis

	Source of Fetal Liver Ribosomes (^{14}C)leucine Incorporated (pmol/30 min/mg protein)	
Source of Fetal Liver pH5-Enzyme	Control	Ethanol-Fed
Control	110 ± 9 (8)	86 ± 7 (8)
	$p<0.02$	$p<0.02$
Ethanol-fed	90 ± 8 (8)	60 ± 5 (8)

SOURCE: Rawat, *Biochem J,* 160:653-661, 1976, by permission. Copyright 1976 by The Biochemical Society, London.

Note: Hepatic pH5-enzyme fractions and ribosomes were prepared from the fetuses from control or ethanol-fed rats. The rates of (^{14}C)leucine incorporation by hepatic ribosomes from both control and ethanol-fed groups were studied in the presence of hepatic pH5-enzyme fractions from fetuses of control and ethanol-fed groups. The details of the experimental procedure are given in the materials and methods section. The results are expressed as means ± SEM, with the number of observations in parentheses.

Table 7. Effect of Mixing Various Heart Fractions From Chronic Ethanol-Fed and Control Rats on Protein Synthesis

Source of Cardiac pH5-Enzyme	Source of Cardiac Ribosomes	
	Sucrose	Ethanol
	(pmol (^{14}C)leucine incorporated/min/mg protein)	
Sucrose-fed	123 ± 10 (6)	105 ± 8 (6)
Ethanol-fed	100 ± 7 (6)	62 ± 8 (6)

Note: Cardiac pH5-enzyme fractions and ribosomes were prepared from sucrose- and ethanol-fed rats. The rates of (^{14}C)leucine incorporation by ribosomes from both control and ethanol groups were studied in the presence of pH5-enzyme fractions from either control or ethanol groups. The results are expressed as mean ± SEM, with the number of observations in parentheses.

Table 8. Effects of Prolonged Maternal Ethanol Consumption on the Rates of Protein Synthesis by Ribosomes and pH5-Enzyme Fraction From Fetal and Neonatal Hearts

	Rate of ($U^{14}C$)Leucine Incorporation	
	Ribosomal + pH5-Enzyme Fraction (pmol/min/mg protein)	
Treatment	Fetal	Neonatal
Sucrose	28.6 ± 4.1 (8)	96.0 ± 8.0 (8)
Ethanol	19.3 ± 3.0 (8)	70.4 ± 6.0 (8)

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Note: Effects of prolonged maternal ethanol consumption were investigated on the rates of protein synthesis by ribosomal and pH5-enzyme fractions from rat fetal and suckling neonatal hearts. The details of ribosomal and pH5-enzyme fraction preparation and incorporation of ($U^{14}C$)leucine into their proteins are given in the section on materials and methods. The results are expressed as mean ± SEM, with the number of experiments in parentheses.

Table 9. Effects of In Vitro Addition of Ethanol or Acetaldehyde on the Rates of ($U^{14}C$)Leucine Incorporation Into Fetal and Neonatal Heart Proteins

	Rate of ($U^{14}C$)Leucine Incorporation Into Cardiac Proteins			
Age	Nil	Ethanol (10 mM)		Acetaldehyde (2 mM)
Fetus	565 ± 53	543 ± 38	N.S.	505 ± 50 (6)
Neonate	905 ± 85	875 ± 68	N.S.	668 ± 70 (6)

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Note: Effects of in vitro addition of ethanol (10 mM) or acetaldehyde (2 mM) were investigated on the rates of ($U^{14}C$)leucine incorporation into cardiac proteins in whole homogenates of rat fetal and neonatel hearts. The details of the protein synthesis determination are given in the text. The results are expressed as mean ± SEM, with the number of experiments in parentheses.

Table 10. Effects of Acetaldehyde or Ethanol Addition on the Rate of Cardiac Protein Synthesis

Additions	Rate of Cardiac Protein Synthesis (pmol/30 min/mg heart protein)	
	Whole Homogenate	Heart Slices
Nil	701 ± 60 (8)	750 ± 65 (10)
Ethanol (10 mM)	685 ± 55 (8)	635 ± 60 (10)
Pyrazole (2 mM)	678 ± 53 (8)	630 ± 60 (10)
Ethanol + Pyrazole (10 mM) (2 mM)	668 ± 50 (8)	600 ± 60 (10)
Acetaldehyde (1 mM)	403 ± 38 (8)	468 ± 40 (10)
Disulfiram (2 mM)	700 ± 40 (8)	765 ± 38 (10)
Acetaldehyde + Disulfiram (1 mM) (2 mM)	575 ± 50 (8)	590 ± 50 (10)
Cycloheximide (2 mM)	175 ± 20 (8)	180 ± 25 (10)

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Note: Effects of in vitro addition of ethanol (10 mM) or acetaldehyde (1 mM) were investigated on the rates of ($U^{14}C$)leucine incorporation into cardiac proteins in whole homogenate and cardiac slice preparations. In experiments where the effect of inhibitors was studied, pyrazole (2 mM) or disulfiram (2 mM) was added. The incubation was carried out at 37°C for 30 minutes and the incorporation of ($U^{14}C$)leucine was studied, as described in the text. The results are expressed as mean ± SEM, with the number of observations in parentheses.

Table 13. Effect of Maternal Ethanol Consumption on Fetal Hepatic RNA, DNA, and Protein Contents

Treatment Group	Total RNA (mg/g fresh wt. liver)	Total DNA (mg/g fresh wt. liver)	RNA/DNA	Total Protein (mg/g fresh wt. liver)	RNA/Protein	Ribosomal Protein (mg/g fresh wt. liver)
Control	5.00 ± 0.25	1.90 ± 0.20	2.63	95.85 ± 5.0	0.052	0.75 ± 0.1
	$p<0.02$	N.S.	$p<0.02$	$p<0.02$	N.S.	$p<0.02$
Ethanol	3.80 ± 0.20	1.93 ± 0.20	1.96	86.70 ± 5.0	0.044	0.53 ± 0.1

SOURCE: Rawat, *Biochem J,* 160:653-661, 1976 by permission. Copyright 1976 by The Biochemical Society, London.

Note: Effect of prolonged ethanol consumption by pregnant rats was investigated on the fetal hepatic contents of total RNA, total DNA, and total protein. The livers were homogenized in 0.25 M sucrose. The ribosomal fractions were prepared as described in the text. The whole liver fractions or ribosomes were taken for the extraction of RNA or DNA. The details of RNA and DNA determinations are given in the materials and methods section. The values are expressed as means ± SEM for six observations. N.S. = not significant.

Table 12. Effect of Prolonged Maternal Ethanol Consumption on the Proteolytic Activity of Fetal and Neonatal Hearts

	Cardiac Proteolytic Activity (percent of sucrose neonate)	
Age	Sucrose	Ethanol
Fetal	75.0 ± 8.0 (6)	70.8 ± 7.0 (6)
Neonatal	100.1 ± 6.0 (6)	82.6 ± 9.0 (6)

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Note: Effects of prolonged maternal ethanol consumption were investigated on the proteolytic activity in the hearts of rat fetuses and neonates. Hearts from fetuses and neonates were homogenized and proteolytic activity was determined against casein as substrate of pH 7.5. The results are expressed as mean ± SEM, with the number of experiments in parentheses.

Table 11. Effect of Ethanol Consumption on the Development of Proteolytic Activity in Rat Liver

Age	Treatment	Hepatic Proteolytic Activity (percent of adult control)	
Adult	Control	100.0 ± 9.0 (8)	
			$p<0.02$
	Ethanol	83.6 ± 7.0 (8)	
Newborn	Control	85.5 ± 6.3 (8)	
			$p<0.02$
	Ethanol	68.5 ± 5.2 (8)	
Fetus	Control	70.5 ± 6.3 (12)	
			$p<0.001$
	Ethanol	48.5 ± 5.2 (12)	

SOURCE: Rawat, *Biochem J,* 160:653-661, 1976, by permission. Copyright 1976 by The Biochemical Society, London.

Note: Effect of ethanol consumption was investigated on the hepatic proteolytic activity of the adult rat, and the effect of maternal ethanol consumption was investigated on the proteolytic activity of newborn rats and fetuses. Liver homogenates were incubated with hemoglobin in a buffered medium (pH 7.6). The details of the procedure are given in the materials and methods section. The results are expressed as percentages of activity of the adult control. The values are means ± SEM, with the number of observations in parentheses.

Table 14. Effects of Prolonged Maternal Ethanol Consumption on the Content of Total Proteins, RNA, and DNA in the Fetal and Neonatal Hearts

		Cardiac Constitutes		
Treatment	Age	Total Proteins (mg/g wet heart)	RNA (mg/g wet heart)	DNA (mg/g wet heart)
Sucrose	Fetus	81.0 ± 9.4	0.99 ± 0.10	3.1 ± 0.40 (6)
	Neonate	96.0 ± 10.6	1.70 ± 0.40	4.50 ± 0.48 (6)
Ethanol	Fetus	71.0 ± 8.0	0.80 ± 0.08	2.89 ± 0.09 (6)
	Neonate	90.1 ± 11.1	1.26 ± 0.30	4.39 ± 0.60 (6)

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Note: Effects of prolonged maternal ethanol consumption were studied on the cardiac content of total proteins, RNA, and DNA. The details of experimental procedures are given in the section on materials and methods. The results are expressed as mean ± SEM, with the number of experiments in parentheses.

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Trends in Alcohol and Protein Metabolic Research*

Marcus A. Rothschild, Murray Oratz, Jorg Morland, and Sidney S. Schreiber

Abstract

Although it is clear that ethanol exposure results in an altered apparent protein synthetic rate, the specific effects on protein secretion are still in doubt. Glycoprotein secretion by EtOH appears to be impaired, but effects on the secretion of other proteins such as albumin are less clear. To study this point, techniques must be used in which synthesis is permitted to proceed prior to the addition of ethanol. Cells were incubated with (^{3}H)valine for 2.5 to 7.5 minutes followed by a 15 mM valine chase, and the incubates were adjusted to final concentrations of ethanol of 50 mM, 100 mM, colchicine 5 to 50 μM, or cycloheximide 18 μM. Cells and media were harvested at various times, counts were incorporated into medium, and cell protein was determined. Cycloheximide inhibited protein synthesis by 99 percent, decreased protein secretion by 10 to 20 percent, but did not further inhibit protein labeling when given following the chase confirming the chase's effectiveness. Colchicine inhibited protein release by 27 to 54 percent depending on the dose. With control cells, labeled protein and specifically albumin appeared in the medium 20 minutes from the start of the pulse, and this release of protein was not inhibited by 50 mM or 100 mM ethanol incubated with cells from the same animal and if the donor had been fed or fasted. The values for the ethanol-treated cells ranged from 94 to 113 percent of the control values for 30 to 120 minutes following the addition of the pulse. Lactate levels were markedly elevated and urea synthesis decreased in the presence of either 50 mM EtOH or 100 mM EtOH. Thus, using a method that can distinguish the effect of ethanol on synthesis from secretion, it is concluded that acute exposure to EtOH does not interfere with protein secretion. Using rats exposed to an ethanol-

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containing diet from 2 to 9 weeks as donors, protein secretion was studied in a similar fashion as described above. The rates of protein secretion from the EtOH-incubated cells compared to control cells in 11 studies were at 30 minutes, 92 ± 9 percent; 60 minutes, 101 ± 4 percent; and 90 minutes, 104 ± 8 percent after pulsing. Urea synthesis in hepatocytes exposed to EtOH was 69 percent ± 6 percent of the control rate. Lactate increased by 0.39 μmol ± 0.08/ml incubate compared to controls. The lack of effect of EtOH on the secretion of rapidly labeled protein in hepatocytes following prolonged in vivo EtOH exposure, although similar to that reported for hepatocytes from rats not exposed to EtOH, contrasts with studies where labeling was prolonged (30 to 60 minutes). It is interesting to speculate that rapidly labeled proteins take a secretory route less sensitive to EtOH, whereas proteins synthesized at a slower rate have a slightly different secretory process more susceptible to EtOH.

The effects of ethanol, acetaldehyde, and their metabolic products on protein synthesis are being actively studied, and the results so far have raised many new thoughts.

Apparent protein synthesis, namely production plus output by the liver, is lowered during exposure to ethanol, although this finding does not apply to all proteins. The hepatomegaly associated with chronic ethanol intake in experimental animals is associated with considerable nitrogen retention, and although albumin and transferrin are increased within the cell (probably as precursor molecules), these two major exportable proteins account for less than 1.5 percent of the accumulated excess nitrogen retained during chronic ethanol exposure (Baraona et al. 1977; Morland and Bessesen 1977; Morland et al. 1979). In other studies employing liver slice techniques, inhibition of both glycoprotein secretion and synthesis has been reported following ethanol ingestion in experimental animals. Thus, the question of whether ethanol affects protein synthesis or secretion remains to be resolved (Tuma et al. 1980).

In our own laboratory, we have examined apparent albumin synthesis using the isolated perfused liver and albumin secretion using rat hepatocytes obtained from fed and fasted rats. Employing the isolated perfused rabbit liver, we have examined the effects of exposing the liver to 200 mg% ethanol for 75 minutes prior to the addition of the label and then following albumin synthesis by measuring the hepata albumin output in the perfusate 2.5 hours later, at a time when all newly synthesized albumin would have been released from the liver. We have called this synthesis in the past, but truly it represents synthesis plus secretion.

^{14}C-CO_2 was used as the label. The guanidino carbon of arginine is labeled along with other carbons. However, this carbon is the

carbon of urea, and hence it is necessary to measure only the specific activity of the newly synthesized urea C to determine the precursor specific activity of the arginine guanidino C that entered albumin. The guanidino C from albumin is also isolated and its specific activity determined. This concept has been validated by simultaneous immunologic measurements of albumin synthesis under a variety of experimental conditions (Oratz et al. 1978; Rothschild et al. 1974).

Using livers from fed donors, albumin synthesis averages 22 ± 1 mg/100 g wet liver weight per hour; fasting reduces this rate to about one-half the fed control value. Employing 10 mM levels of certain but not all amino acids, this fasting decrease in albumin synthesis can be reversed, and the associated disaggregation of the endoplasmic membrane bound polysome is also reversed (Rothschild et al. 1968).

Acute exposure to ethanol likewise reduces albumin synthesis in livers from fed donors, and the bound polysomes are also disaggregated. The same amino acids that were effective in reversing the fasting effects likewise reversed the acute effects of ethanol. It was this observation that led us to suggest that acute ethanol exposure acted like a pharmacologic fast, with the exception that hepatic RNA was not reduced during ethanol exposure.

When both stresses are applied together—namely, fasting plus ethanol— albumin synthesis is reduced only a small degree further than with either stress alone, but now the free polysome is also disaggregated and the amino acids that previously had been effective in reversing the effects of either stress applied singly were no longer active (Oratz et al. 1978; Rothschild et al. 1974).

We next investigated the role of alcohol metabolism in these results and whether acetaldehyde underlay these results. Again with livers from fed donors, the inhibition of ethanol oxidation with 1.5 mM 4-methyl pyrazole did not improve the rate of albumin synthesis but did prevent much of the polysome disaggregation. When fasted donors were used, 4-MP did improve albumin synthesis, although the change was small and polysome aggregation remained at the level seen with fasting alone (Oratz et al. 1978). Acetaldehyde 2 mg% reduced albumin synthesis in livers from fed donors and also lowered urea synthesis, but bound polysomes were unaffected. In the fasting state, acetaldehyde different from ethanol had no effect whatsoever. When acetaldehyde metabolism was blocked with 4-MP plus disulfiram, the acute effects of

acetaldehyde on albumin metabolism in livers from fed donors were reversed.

Thus, these observations raised more questions—obviously all the effects of ethanol exposure could not be explained by acetaldehyde per se; polysome disaggregation appeared to be related to ethanol metabolism, not to ethanol or acetaldehyde per se or to acetaldehyde metabolism, and the absolute state of hepatic nutrition governed the type of reaction. In other studies using the perfused heart as the model, it was observed that although acetaldehyde was toxic to protein synthesis, polysomes were not disaggregated (Schreiber et al. 1972). Thus the effects of ethanol exposure on albumin synthesis are complex and not susceptible to a clear, unified concept as yet.

We then turned to examine the effects of ethanol on protein and albumin secretion. To study secretion separate from synthesis, it is necessary to permit synthesis to proceed in the absence of the test substance that may affect both processes. Thus rat hepatocytes were pulse labeled with (^{3}H)valine for 2.5 to 7.5 minutes; the labeling was then essentially inhibited completely by a 15 mM valine chase, and the rate of secretion of total protein and albumin was studied in the presence and absence of ethanol. No effect of ethanol on secretion was noted at either 50 or 100 mM levels while lactate levels rose and urea synthesis was depressed. Thus our studies failed to confirm other observations indicating a specific defect in export protein secretion (Morland et al. 1981).

Again, as with specific protein synthesis, protein secretion may not be uniformly affected by ethanol or its metabolic pathway. Perhaps those proteins whose synthesis time is short are affected in a different way than are those requiring a longer schema. Where ethanol operates in the synthetic mechanism is not known. The persistent polysome disaggregation seen suggests an early step, but data are not available. Further speculation is not warranted now, but the field is just recently being studied in depth, and our ideas will change rapidly within the next few years.

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Effect of Ethanol on Macromolecular Synthesis

Jean Swedes and Calvin S. McLaughlin

As a model single cell system for the study of the molecular basis for ethanol toxicity we are using the eukaryotic organism *Saccharomyces cerevisiae*. We find that protein synthesis is inhibited, increasing as the ethanol concentration is varied over the range of 1 to 10 percent (v/v). The ethanol concentration required to produce 50 percent inhibition of protein synthesis is altered depending on the growth medium of the cells. The ethanol values were 1.8, 4.4, and 5.2 percent, respectively, for cells grown in minimal medium, minimal medium plus 19 amino acids, and complex medium. Polysome metabolism was altered upon addition of ethanol. The amount of polysomes declined and monosomes increased. Taken together, our data from intact cells suggest that protein synthesis is inhibited by ethanol (or a derivative) at both an elongation site and an initiation site of polypeptide synthesis.

We are investigating the mechanism by which ethanol inhibits the cell's protein synthesizing system. An interference with normal energy metabolism is one possibility. We find a decline in the ATP/ADP and GTP/GDP ratio upon addition of ethanol. This may be part of the molecular basis for inhibition.

One advantage of using *S. cerevisiae* for investigating the mechanism of action of a toxin such as ethanol is the ease of genetic manipulation. We have available mutants with a wide range of alcohol dehydrogenase activities, including strains M63 and SF1-1C-M3, with 1 percent or less of wild type activity; and strains 11-13C-R1, R234, R22, which have 300 to 400 percent of wild type activity. We are investigating the relative sensitivities of these strains to ethanol.

We have looked at the rate of synthesis of individual proteins using the O'Farrell gel system. After ethanol addition we found that a few proteins (about 10) are synthesized at very elevated levels. These same proteins are made when the cell is put under other types of stress, e.g., heat shock and energy inhibitors. The synthesis of these proteins may be part of a general stress response.

We are following up these in vivo experiments using an in vitro protein synthesizing system. The ethanol concentration required for inhibition of the in vitro system is 5 to 7 percent ethanol (v/v) in the same range as in vivo. This in vitro system is a powerful tool for determining the mechanism of ethanol's effect on protein synthesis. We will be able to localize the steps in polypeptide synthesis inhibited by ethanol. Furthermore, with the in vitro system and temperature-sensitive mutants available to us, we will be able to distinguish between translational and transcriptional control of alcohol—or stress-induced proteins. [Paper was not available for inclusion in the text of this Monograph.]

Ethanol and Peptidyl Puromycin Reaction in Brain

Sujata Tewari

Effects of chronic ethanol ingestion by adult male Sprague-Dawley rats were determined on the synthesis of peptidyl-puromycin on brain ribosomes. Data revealed that, although isolated brain ribosomes from the control and ethanol groups were highly active in the in vitro incorporations of (H^3)puromycin into nascent polypeptide chains on brain ribosomes, ethanol ingestion resulted in significant stimulation of the transpeptidation reaction. Under these conditions, the reaction was completely dependent on ribosomes and was anisomycin sensitive. Data further confirmed the multiple effects of ethanol on the brain protein synthesizing system and demonstrated that the target sites are on ribosomes whose specific properties were altered by ethanol. Experiments carried out on 80S ribosomes or ribosomal extracts showed results similar to those obtained with unfractural ribosomes. The observed increase in the synthesis of peptidyl-puromycin could be due to the activation of the dormant peptidyl-transferase reaction and/or a specific effect on the P site on ribosomes. [Paper was not available for inclusion in the text of this Monograph.]

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Protein Synthesis After Ethanol Administration to Isolated Hepatocytes Derived From Fed and Fasted Rats

Jorg Morland

Abstract

Hepatocytes from either fed or fasted (10 to 14 hours) rats were isolated and incubated in the presence and absence of ethanol, 10 mM. In cells from fasted animals, ethanol inhibited protein synthesis by approximately 20 percent both in the presence of physiological amino acid concentrations and in the presence of amino acid levels eight times higher. Ethanol did not inhibit protein synthesis in cells derived from fed animals. Previous feeding increased protein synthesis in hepatocytes by approximately 30 percent compared to liver cells from fasted animals. This effect was absent at high amino acid levels. It was concluded that ethanol inhibits protein synthesis in hepatocytes from fasted animals only, by a mechanism not directly linked to its effect on amino acid metabolism.

Introduction

Suspensions of isolated cells may conveniently be used to study protein synthesis because the rate of this process may be measured at controlled conditions more easily in isolated cells than in vivo. It has previously been shown that the addition of ethanol inhibits the incorporation of labeled valine into hepatocyte proteins (Morland and Bessesen 1977; Morland et al. 1979, 1980), and we have concluded that this effect is due to reduced protein synthesis

NOTE: Tables appear at end of paper.

(Morland and Bessesen 1977). Such experiments have been carried out on liver cells from 10- to 14-hour fasted rats. Because fasting by itself influences hepatic protein metabolism (Garlick et al. 1975; Wunner et al. 1966), it is possible that the effect of ethanol on liver protein synthesis could be dependent on the nutritional state of the organism. During chronic ethanol consumption, the combination of the stresses of fasting and ethanol will often occur. It was therefore of interest to see whether this combination in an isolated liver cell system would lead to results different from those obtained after ethanol in hepatocytes derived from well-nourished animals.

Materials and Methods

Animals

Male Wistar rats, weighing 200 to 250 g, were maintained on a 12-hour light/12-hour dark cycle. Some rats were fasted for 10 to 14 hours before the experiments, while others were fed until preparation of liver cells.

Cell Preparation and Incubation

Parenchymal rat liver cells were prepared after perfusion with a Ca^{++}-free buffer followed by a collagenase solution according to Berry and Friend (1969) and Seglen (1973), as described previously (Berg and Morland 1975; Morland et al. 1980). Washed parenchymal cells were suspended and incubated in a buffered salt solution containing glucose (5.5 mM), antibiotics, albumin (2 percent), and a mixture of amino acids. The composition of the medium was as described previously (Morland et al. 1979) except that 20 amino acids (Morland and Bessesen 1977) were present at concentrations equal to the physiological level for the rat. In some experiments, 20 amino acids (Morland and Bessesen 1977) were added to a final concentration eight times the physiological level, whereas in other experiments no amino acids were present. Incubation of 2.5×10^6 viable cells per milliliter was carried out at 37°C usually for 1 hour. Control and experimental cell suspensions (30 to 40 ml) derived from the same liver were incubated in parallel in stoppered 1 l flasks.

L-(U-^{14}C)valine (CFB.75, 270 Ci/mol), final concentration 0.05 μCi, was added after 1 minute. Ethanol was added after 15 minutes as a small volume of a 10 percent (v/v) solution in 0.9 percent saline to a final initial concentration of 10 mM. Otherwise the incubations were performed as described (Berg and Morland 1975; Morland and Bessesen 1977; Morland et al. 1980).

Analytical

Samples of cell suspension were analyzed for cell number and viability (trypan blue exclusion test). Other samples were centrifuged at 1,600 g for 1 minute, and the media were analyzed for ethanol concentration (F42 Perkin Elmer Head Space Gas Chromatograph) and frozen at -20°C, as were the cell pellets. Radioactivity incorporated into proteins was determined in frozen samples as described previously (Morland 1974), and protein concentration was determined according to Lowry et al. (1951). Free amino acid concentrations were measured by means of a Jeol JLC-6AH amino acid analyzer. The radioactivity of free valine was measured after collecting a part of the splitted eluate from the analyzer. The specific radioactivity of valine was determined by dividing the radioactivity of the valine peak by the amount of total free valine. Radioactivity was determined with a Packard Tri-Carb 3320 scintillation spectrometer, and the samples were tested for quenching by the channels-ratio method.

Calculation of Protein Synthesis

In experiments with physiological amino acid concentrations, the concentrations of free labeled and unlabeled valine in the intracellular and extracellular compartment were calculated from measurements in cell pellets and media (Morland et al. 1979). The rate of protein synthesis was then calculated from the mean specific radioactivity of valyl t-RNA calculated according to Vidrich et al. (1977). In experiments with high amino acid levels, the specific radioactivity of the precursor was calculated from the amounts of radioactive and unlabeled valine added plus the endogenous level present. Protein synthesis was then calculated on this basis. Wilcoxon's test for paired comparisons was used to compare results in hepatocytes derived from the same liver.

Results

Protein synthesis was inhibited by ethanol in hepatocytes from fasted rats (table 1). The inhibition was present both for cellular proteins and for proteins secreted to the media. The mean protein synthetic rate decreased to about 80 percent of the control rate. Ethanol exerted a similar effect when protein synthesis was measured in an experiment with physiological amino acid concentrations and high valine concentration (approximately 2.5 mM). A similar effect of ethanol was present when the experiment was performed in the absence of amino acids except valine (5 mM) in the media (data not shown). The effect of ethanol was independent of the anesthetic used during liver cell preparation. The inhibition of protein synthesis disappeared when cells incubated with ethanol were washed and reincubated in fresh medium (data not shown). Feeding the liver cell donors increased the mean control protein synthetic rate by approximately 30 percent (compare lines 1 and 3 in table 1). Feeding also rendered hepatocyte protein synthesis less susceptible to ethanol. No effect of ethanol on cellular or total protein synthesis was observed in preparations from fed rats (table 1). However, a small effect of ethanol on medium protein synthesis persisted (table 1).

There was no difference between hepatocytes from fasted and fed rats with respect to initial and final ethanol concentration, pH reduction due to ethanol metabolism (0.16 $\pm$ 0.01 pH units in fasted and 0.14 $\pm$ 0.03 pH units in fed cells), initial and final viability (94.8 $\pm$ 3.2 percent and 92.2 $\pm$ 2.4 percent), concentration of labeled valine, or specific valine radioactivity. Viability, valine concentration, and specific radioactivity were not altered in the presence of ethanol.

It has been demonstrated that ethanol may interfere with the cellular uptake of amino acids (Chambers and Piccirillo 1973; Piccirillo and Chambers 1976) and intracellular amino acid metabolism (Häkkinen and Kulonen 1975; Krebs et al. 1973). This could constitute a basis for the action of ethanol on protein synthesis through a relative lack of one or more amino acids, which again could depend on the nutritional state. Since not all amino acids could be measured by the amino acid analyzer at the conditions used (which were aimed particularly at valine determination), and since the low levels of tryptophan and arginine are difficult to detect, we increased the levels of all amino acids to approximately

eight times the physiological levels in another series of experiments. This procedure would reduce any effect on protein synthesis mediated through amino acid depletion. Table 2, however, shows that the effect of ethanol was still present in cells from fasted rats. By comparison with table 1, it is clear that supraphysiological levels of amino acids could not relieve the inhibition of protein synthesis caused by ethanol, although some stimulation of protein synthesis was found in control cells. In the presence of eight times the physiological amino acid level, there was no substantial difference in protein synthesis between control cells from fasted and fed animals (table 2), but still the hepatocytes from fed donors were protected against the inhibitory effect of ethanol.

Again, no differences between hepatocytes from fasted and fed rats as well as between control and ethanol-treated cells were observed with respect to viability, valine concentration, and specific radioactivity.

Discussion

In accordance with previous results (Morland and Bessesen 1977; Morland et al. 1979, 1980), ethanol inhibited protein synthesis in rat hepatocytes. This inhibition did not appear to be mediated via changed amino acid levels because the effect of ethanol was as marked in the presence of eight times physiological amino acid levels. The latter finding is in accordance with studies on albumin synthesis in the intact perfused fasted rabbit liver. In that system, it was shown that the acute depression of albumin synthesis caused by ethanol could not be reversed by supraphysiological levels of amino acids (Rothschild et al. 1974). The mechanism underlying the acute effect of ethanol in these systems is mainly unknown.

A different susceptibility to ethanol was found in cells derived from fed and fasted rats. The higher resistance of the former cell type against ethanol's inhibition of protein synthesis is in accordance with earlier work on albumin synthesis (Rothschild et al. 1974). In those studies it was demonstrated that the effect of ethanol could be reduced by addition of amino acids to fed livers but not when added to livers from fasted animals. In the presence of physiologic amino acid levels, we found that previous feeding by itself increased protein synthesis by approximately 30 percent. This result is consistent with other reports (Henshaw et al. 1971; Hirsch

1976; Hirsch and Hiatt 1966; Munro et al. 1953; Sox and Hoagland 1966), although more dramatic quantitative differences than the present one have been reported. The relatively short fasting period as well as the use of the same incubation medium for fed and fasted cells in our experiments may explain this difference. The difference between fed and fasted control cells was not evident in the presence of eight times physiological amino acid levels. Thus, amino acids could reverse the effect of fasting on protein synthesis in our system.

Despite this restoration of protein synthetic rates, fasted cells were more susceptible to the inhibitory effect of ethanol. We have recently found the same phenomenon in hepatocytes derived from human liver (Gustavsson et al. 1980). It could be postulated that cells isolated from a fasted organism were inferior to cells from a fed rat due to lower resistance to anesthesia, operation, and cell isolation. However, our results on cell viability, amino acid levels, and protein synthetic rates, when stimulated by high amino acid levels, are against this explanation. A more specific cause could be that fasted cells metabolized ethanol more slowly, pointing at a direct effect of ethanol per se on protein synthesis. In an experimental system like the present one, such differences would result in barely detectable differences in actual ethanol concentration, which makes this explanation less likely. It has been demonstrated that the polysomes are more disaggregated in hepatocytes from fasted rats than from fed rats (Dickson and Pogson 1980). It could therefore be postulated that protein synthesis was more susceptible to ethanol when the polysome profile displayed a disaggregated pattern. To check this, two different cell preparations derived from the same fasted liver were compared. In one, the polysomes were extensively disaggregated by repeated washing of the cells. In this preparation, ethanol inhibited protein synthesis by 7 percent. In the other preparation, the polysomes were much more aggregated, and ethanol inhibited protein synthesis by 35 percent. Thus any polysome disaggregation caused by fasting very unlikely rendered the cells more susceptible to the inhibitory action of ethanol. A series of other biochemical differences have been found in livers of fed and fasted animals. It is, however, at present difficult to link differences, e.g., with respect to drug detoxification and gluthathion levels, to the present observation on ethanol inhibition of protein synthesis.

Further, it is at present possible only by speculation to link our observation to in vivo effects of ethanol on liver protein synthesis. If

there is a similar inhibition in vivo and if this inhibition represents early liver damage, a modulating role for nutrition in the development of alcoholic liver injury should be looked for more closely in future experiments.

Table 1. Effect of Ethanol on Protein Synthesis in Hepatocytes From Fasted and Fed Rats

	Protein Synthesis (nmol/mg cell protein)			Mean Protein Synthetic Rate (pmol/mg cell protein/min)
	Cell	Medium	Total	Total
Fasted				
Control	2.87 ± 0.24	0.54 ± 0.14	3.42 ± 0.37	78 ± 9
Ethanol	2.23 ± 0.34[a]	0.34 ± 0.16[a]	2.74 ± 0.48[a]	62 ± 11[a]
Fed				
Control	3.66 ± 0.30	0.75 ± 0.10	4.41 ± 0.39	100 ± 9
Ethanol	3.63 ± 0.35	0.66 ± 0.08[a]	4.29 ± 0.41	98 ± 9

[a] Statistically significantly different from respective control value, $\alpha < 0.05$.

Note: Cells were incubated for 1 hour in media containing physiological amino acid levels. Ethanol or saline was added after 15 minutes, and protein synthesis during the subsequent 45 minutes was calculated. Results from six experiments are given ± SEM.

Table 2. Effect of Ethanol on Protein Synthesis in Hepatocytes From Fasted and Fed Rats

	Protein Synthesis (nmol/mg cell protein)			Mean Protein Synthetic Rate (pmol/mg cell protein/min)
	Cell	Medium	Total	Total
Fasted				
Control	3.34 ± 0.17	0.66 ± 0.23	4.00 ± 0.22	91 ± 5
Ethanol	2.34 ± 0.19	0.39 ± 0.18[a]	2.73 ± 0.14[a]	62 ± 3[a]
Fed				
Control	3.45 ± 0.14	0.78 ± 0.10	4.23 ± 0.15	96 ± 4
Ethanol	3.23 ± 0.22	0.69 ± 0.15	3.91 ± 0.08	89 ± 2

[a] Statistically significantly different from respective control value, $\alpha < 0.05$.

Note: Cells were incubated for 1 hour in media containing eight times physiological amino acid levels. Ethanol or saline was added after 15 minutes and protein synthesis during the subsequent 45 minutes was calculated. Results from six experiments are given ± SEM.

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Effects of Ethanol on the Course of Herpes Simplex Virus Type 1 Replication in Cultured Cells*

B. Gayathri Devi, Kevin P. Anderson, and Edward K. Wagner

Abstract

Over the past several years, we have developed techniques for the isolation of intact, biologically active herpes simplex type 1 (HSV-1) mRNA. We have thus been able to examine in some detail the types of mRNA and the polypetides they encode, which become abundant at various stages of the normal infection cycle of the virus and which have specific functions in the life cycle of the virus. These techniques are also of value in studying the effects of environmental agents that perturb the normal development and pathogenesis of HSV-1. Ethanol is a strong candidate for such an agent because of its known effects on macromolecular metabolism in cells of neural origin and because of HSV-1's strong tropism toward neural tissue in vivo. We have screened the effect of ethanol on virus production, virus cytopathogenicity, and virus-induced mRNA and protein synthetic patterns. We find that cocultivation with 1.5 to 2 percent ethanol reduces virus yield and is marginally cytotoxic to cells. Lower levels of ethanol (around 0.25 percent) can lead to some reduction in amounts of viral DNA synthesized, although not production of infectious virus. No effect on any one specific viral gene produce has been seen. We feel that further studies should be aimed at investigation of specific aspects of viral pathology in vivo before other molecular assays are practicable.

NOTE: Figures and table appear at end of paper.

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Introduction

Natural History of Herpes Simplex Virus Infection

Herpes simplex virus (HSV, *H. hominus*) is a well-characterized member of the herpesvirus group. These large, nuclear-replicating DNA viruses are characterized by their highly evolved relationship with their hosts and by their ability to remain associated with their hosts in spite of normal host defense mechanisms. HSV occurs in two distinct subtypes: type 1 (HSV-1), associated with facial lesions; and type 2 (HSV-2), associated with genital infection. The two types can cross-react antigenically, and their genomes are partially homologous (Nahmias and Roizman 1973; Wildy 1973). The HSV virion comprises as many as 50 distinct polypeptides (Honess and Roizman 1973) arranged in a defined series of layers and shells. The viral DNA is encapsulated in the core of the virion associated with one or several proteins. These are probably involved in binding the viral DNA to facilitate the encapsulation process (Bayliss et al. 1975; Gibson and Roizman 1972). The viral capsid is surrounded by a glycoprotein inner envelope and a lipid-rich membrane. It is known that the viral DNA itself is infectious (Sheldrick et al. 1973); therefore, none of the virion proteins and derivations can be said to be absolutely essential for replication.

In humans, the virus has a remarkable course of infection in that initial infection is followed by recurrent sporadic recrudescence due to the virus' being maintained in a latent state in the host. This latency is due to the virus' maintaining itself in a noninfectious form in the nervous system of the host (see Stevens 1975). The frequency of recurrent eruptions is correlated with the general state of health of the host. Individuals under emotional and physiological stress are often the most susceptible to recurrences. Although infections are usually localized, there is a large literature of disseminated herpetic infections ranging from those that are generalized to herpetic encephalitis (Tokumaru 1969). Indeed, the proclivity for HSVs spreading to the central nervous system in experimental animals is well known (Darlington and Karnoff 1973) and has led to animal models for HSV latency (Stevens and Cook 1973). In fact, the presence of HSV in the brain has been implicated in certain psychopathic states (Cleobury et al. 1971). The role of the state of health of the host in such atypical HSV infections is unclear.

Replication Cycle of HSV in Cultured Cells

In the laboratory, HSV-1 grows very well in continuous line cultural human cells such as HeLa cells. Virus infection results in cell death, but the cells do not lyse; rather, they either clump up or fuse together, depending upon the strain of virus used. In such cells, the infectious cycle is quite rapid; and at moderate multiplicities of infection (MOI) of 5 to 10 plaque-formed units (PFU) of virus per cell, new infectious virus can be readily detected by 12 to 15 hours after infection. Under the best conditions, an infected HeLa cell can produce as much as 200 PFU of new virus, although 50 to 100 PFU per cell are more usual.

HSV Gene Expression During the Infectious Cycle

HSV-1 DNA is characterized by a high guanine plus cytosine content (68 percent G+C); therefore, it is readily separable from cellular DNA by isopycnic centrifugation, and amounts of viral DNA synthesized in infected cells can readily be measured. In HeLa cells under moderate MOI, viral DNA replication can readily be seen by 4 hours after infection (Wagner 1972).

HSV-1 is characterized by an unusual arrangement of its genome. The viral DNA has a molecular weight of 95 to 100 $\times$ 10^6 daltons (reviewed by Roizman 1979), which corresponds to a length of 150,000 base pairs (150 kb pairs; Wagner et al. 1976). The linear HSV-1 genome is segmented into a long region (ca. 82 percent) and a short region (18 percent), each bounded by different inverse repeat sequences (Wadsworth et al. 1975). Such a situation results in four equimolar populations of the viral DNA differing in the relative orientation of the long and short segments (Hayward et al. 1975; Skare and Summers 1977; Wilkie and Cortini 1976). In view of this fact, one arrangement has, by convention, been chosen as the prototypical (P) configuration (Morse et al. 1978; Roizman 1979). A representation of this arrangement, indicating specific restriction endonuclease cleavage sites, is shown in figure 1.

The size of the HSV-1 genome is large enough to encode a large number of viral polypeptides. If one assumes an average size of viral polypeptide to be in the range of 60,000 daltons (d), then this suggests an average mRNA size of 2,000 bases (2 kb) or so, and the virus is large enough to encompass as many as 75 distinct, nonoverlapping, average-size mRNAs. Although such a number is

only a rough estimate, it does indicate the complexity of HSV genes and gene expression.

We, as well as workers in other laboratories, have been engaged in investigating the properties of HSV-1 mRNA as a general model for herpesvirus mRNA for several years. HSV-1 mRNA shares general properties with host cell mRNA; i.e., it is synthesized in the nucleus, polyadenylated on the 3′ end and capped on the 5′ end, and internally methlyated (Bachenheimer and Roizman 1972; Bartkoski and Roisman 1976; Morse et al. 1977; Silverstein et al. 1976; Stringer et al. 1977; Wagner and Roizman 1969).

It is interesting to note that although HSV-1 mRNA is in many ways like its host cell mRNA, it may differ in one respect—its degree of splicing. RNA splicing appears to be a common feature of mammalian mRNA biogenesis, in which unneeded internal sequences are removed by an as yet uncharacterized mechanism and the resulting mRNA sequences are spliced back together (reviewed by Crick 1979). Although some HSV-1 mRNAs are spliced during this biogenesis (Anderson et al. submitted for publication; Watson et al. in press), many others do not appear to be (Anderson, Holland, Gaylord, and Wagner 1980; Costa et al. submitted for publication). Whether this result is a reflection of the unusual natural history of the virus is, as yet, unclear.

There are three stages of HSV-1 replication, each characterized by an increasing complexity of viral mRNA expressed. The first stage, immediate-early, is comprised of those RNA species that can be expressed abundantly without de novo protein synthesis, i.e., with an unmodified host cell RNA polymerase (Kozak and Roisman 1974; Rakusanova et al. 1971). Abundant members of this class of mRNA are quite limited, map in regions of the HSV-1 genome at or near the long (L_R) and short (S_R) repeat regions, and encode only a limited number of polypeptides in vivo and in vitro (Anderson, Holland, Gaylord, and Wagner 1980; Clements et al. 1977; Holland et al. 1979; Honess and Roizman 1974; Jones et al. 1977; Marsden et al. 1976; Preston 1979*a*,*b*; Watson and Clements 1978; Watson et al. 1979).

Following expression of one or several immediate-early HSV-1 proteins, a more complex population of viral mRNA is abundant prior to viral DNA replication (Huang et al. 1971; Murray et al. 1974; Swanstrom and Wagner 1974; Swanstrom et al. 1975; Wagner 1972; Wagner et al. 1972). This early viral mRNA maps throughout the HSV-1 genome in noncontiguous regions, but only a limited number of readily resolvable species are found (Holland et al. 1979;

Jones and Roizman 1979; Stringer et al. 1978). Many of these early viral mRNAs are involved with priming the cell for viral DNA replication. We and other workers have shown that without viral DNA replication, the early viral mRNA and protein population appears to persist (Powell et al. 1975; Swanstrom and Wagner 1974; Swanstrom et al. 1975; Wagner 1972; Ward and Stevens 1975). Recently, we have shown that in the absence of viral DNA replication, the specific viral mRNA species and the polypeptides they encode in vitro are virtually indistinguishable from kinetically early ones (Holland et al. 1980).

Concomitant with viral DNA replication is the appearance of late HSV-1 mRNA (Wagner 1972; Wagner et al. 1972). These mRNA species encode a large number of polypeptides (Holland et al. 1980), many of which presumably are structual proteins of the virion.

The size and complexity of the HSV-1 genome have made the detailed characterization of individual HSV-1 mRNA species difficult. We have developed methods for the preparative isolation of biologically active HSV-1 mRNA homologous to specific restriction fragments of the viral genome (Anderson et al. 1979; Anderson, Costa, Holland, and Wagner 1980; Anderson, Holland, Gaylord, and Wagner 1980; Holland et al. 1979). We have used such methods to enumerate and map many HSV-1 mRNA species seen at different stages of infection (reviewed in Wagner et al. in press) (see figure 2).

More recently, we have applied recombinant DNA technology to carry out very high resolution studies of HSV-1 mRNA localization and expression (Anderson, Costa, Holland, and Wagner 1980; Anderson et al. submitted for publication; Costa et al. submitted for publication). These studies allow us to ask precise questions regarding the influence of environmental agents on the course of HSV-1 gene expression.

Rationale for Examining the Effect of Ethanol on HSV-1 Infection

Acute ethanol addiction in humans is characterized by a number of readily identifiable clinical and psychological syndromes, some due to nutritional factors and others to the specific effects of ethanol on the biochemical and macromolecular metabolism of the cells and tissues of the alcoholic (Wahlgren and Barry 1970). The development of animal systems has made it possible to carefully investigate the role of both chronic and acute ethanol consumption

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on specific tissues and molecular processes that eliminate nutritional and sampling biases. Thus, it is known that chronic ethanol consumption in rats leads to decreased protein and RNA synthetic activity in the brain (Noble and Tewari 1972, 1975; Tewari and Noble 1974). Further, withdrawal of ethanol from physically addicted rats leads to profound alterations in the macromolecular metabolism of the brain.

The role of alcoholism in the development of atypical courses of infectious disease has not been subject to much investigation (Wahlgren and Barry 1970). Certainly, however, the alteration of biochemical processes in ethanol-consuming subjects may lead to alterations in the development of and recovery from viral diseases. Also, HSV grown in the laboratory is remarkable in its sensitivity to nutritional and metabolic factors of the host cell (Roizman 1969). This fact, and HSV's proclivity for nervous tissue, makes it an interesting model for the study of the effect of ethanol on the course of an infectious disease. Further, as outlined above, the molecular details of its replication are well known, so specific effects of ethanol can be readily measured.

Experimental Plan

Our experimental approach has been straightforward. We have examined aspects of HSV-1 replication in cell culture that might indicate specific susceptibility to the presence of physiological amounts of ethanol. We have a good deal of knowledge concerning the molecular aspects of HSV-1 replication in cell culture and have the techniques available to examine perturbations of these molecular processes. We have, therefore, determined the course of HSV-1 infection in cells of neural origin, examined how ethanol affects such cells, and then specifically examined HSV-1 mRNA expression in infected cells as a gage for specific effects of ethanol.

Results

Course of HSV-1 Infection in Cultured Neural Cells

Production of HSV-1 in Neural Cells

We examined HSV-1 replication in three cell lines of neural origin: a human glioma (COX), a rat glioma (C-6), and a mouse neuroblastoma (D-2) (Rice et al. 1979). A full description of our culturing and virus assay techniques is contained in Rice et al. (1979).

Production of infectious HSV-1 was measured in small cell cultures. All cell lines studied showed efficient adsorption and penetration of the virus when infected at an MOI of 5 PFU/cell. Virus yield was measured at 48 hours postinfection (hpi) at an MOI of 5 PFU/cell. In all cases, the cells were incubated with 0.4 percent human immune serum globulin from 2 to 6 hpi to neutralize any unadsorbed virus and then rinsed several times with fresh medium. Cells were harvested at 24 or 48 hours and assayed. These data are shown in table 1. It is seen that both D-2 and COX cells for 24 hours have an average burst of about 20 PFU HSV-1 per cell in the infected cultures. Examination of the infected cultures showed all cells to have a large cytopathic effect, and no cells could be cultured from these infected cultures. The virus yield from the cell types is 50 to 60 percent of that found for primary human embryonic skin cells (HESM) infected in parallel (table 1) and is not altered if cells are infected at an MOI of 10 PFU/cell or if the cells are infected at 0.5 PFU/cell and allowed to go 72 hours.

In contrast to the normal cytocidal productive infections of the D-2 and COX cells, rat C-6 cells yield very low amounts of HSV-1 (10^{-2} PFU/cell) at 48 hours after infection when infected at 5 PFU/cell followed by rinsing with immune serum globulin as described for the other cells. Further, no obvious cyptopathic effect was observed in the infected C-6 cultures at 48 or 72 hpi. Infected cultures of C-6 cells were maintained and passed at a split ratio of 1:6 when confluent. After 15 to 16 days of culture, the C-6 cells showed increasing cytopathology, culminating after 25 days with massive cell destruction with only 10 to 15 percent of the cells surviving (figure 3). The surviving cells were maintained in culture and continued to grow to confluence with no evident cytopathology for another 15 to 17 days, when cell destruction and cytopathology again became evident. Again, surviving cells were grown with little

evident cytopathology until a third "crisis" occurred at 75 days following the initial infection. The surviving cells at this time were still viable as in the previous stages.

Such a pattern of cell destruction is characteristic of a persistent infection of the C-6 cells with HSV-1. Infectious virus was assayed in parallel cultures at various times during the cytopathic cycle, and these data are also shown in figure 3. At times of observable cell destruction, virus titers were found to be 1-2 $\times$ 10^6 PFU/culture flask compared to 1 $\times$ 10^{-2}-10^{-3} PFU/culture flask when no obvious cytopathology was evident. The surviving cells evidenced a low but measurable amount of HSV-1 until a new crisis phase occurred when the virus titer again again increased several logs.

Effect of Ethanol on HSV-1 Production in Cultured Neural Cells

We examined the growth of cultured COX and HESM cells in the presence of 0.25 percent, 1 percent, 1.5 percent, and 2 percent ethanol added to the growth medium. The cells grew normally in all but the 2 percent ethanol sample for at least two generations, although they did not appear to grow as well after this in the 1.5 percent sample. A level of 2 percent ethanol was marginally cytotoxic to cells and was not used further. When 1 percent or 1.5 percent ethanol was added to the cell culture medium at time of standard infection (MOI of 5 PFU/cell of HESM or COX cells) with HSV-1, the virus yield in 24 hours was marginally but reproducibly reduced by an average of about 50 percent in the COX cells over the untreated control. In the case of the HESM control cells, only a small reduction was seen (table 1). Such a marginal reduction is often seen when the nutritional state of cells is suboptimum. It does suggest, however, that ethanol has a greater effect on HSV-1 replication in neural cells than in skin cells.

In the case of infected or persistently infected C-6 cells, it was found that even 0.5 percent ethanol leads to marked cytotoxicity, and the cells could not be passaged in the presence of ethanol. This establishes that such neuroblastoma cells are very sensitive to levels of ethanol that have little effect on other cell types. We tested the effect of 0.25 percent ethanol on the production of HSV-1 in a persistently infected C-6 cell culture by incubating such cells with ethanol only after passage of an untreated, infected control culture. As was described above, such persistently infected cultures periodi-

cally demonstrate a large amount of cytopathology and the production of a relatively large amount of virus (figure 3). The addition of ethanol to such cultures immediately leads to observable cytopathology of cells one to two passages prior to that seen in the untreated control cultures (figure 3). Virus yield was not notably affected, however. This result suggests that the sensitivity of C-6 cells to ethanol can lead to ethanol's enhancing viral cytopathic effects.

Effect of Ethanol on HSV-1 Gene Expression in Cultured Cells

Effect of Ethanol on Viral Macromolecular Synthesis in Cultured Cells

As we have described in Rice et al. (1979) and in earlier publications (Rice et al. 1976; Stringer et al. 1977), we can rapidly measure the synthesis of HSV-1 specific DNA and RNA in infected cells. The former can be done by taking advantage of the high equilibrium density of HSV-1 DNA compared to cellular DNA in isopycnic CsC1 or NaI density gradients. Viral DNA can be visualized either directly (figure 4) or by use of radioactivity. We found that no viral DNA synthesis could be detected in HSV-1 infected C-6 cells, but it was readily detectable in infected D-2 and COX cells. Interestingly, in the latter two cases, although both cells produced equivalent amounts of virus, the amount of viral DNA detectable in infected D-2 cells was considerably less than in the COX cells. Viral RNA synthesis was assayed by use of a quantitative hybridization technique described in Stringer et al. (1977) and Rice et al. (1979). It suffices here to say that patterns of viral RNA synthesis reflected those of DNA synthesis in the different cell types (Rice et al. 1979).

We examined the effect of ethanol on viral DNA and RNA synthesis in a manner identical to that described above. Concentrations of 0.25 percent and 1 percent ethanol were used in infected COX, D-2, and HESM cells. We found, interestingly, that both concentrations resulted in an approximate loss of 10 percent of viral nucleic acid synthesis. This effect, however, is too small to be considered significant.

HSV-1 efficiently shuts off host cell protein synthesis in many cultured cells. Because of this, viral proteins can be readily visualized in infected cells. A typical experimental protocol is to

infect cells at a moderate MOI (10 PFU/cell), and after 6 hours add radioactive amino acids to the cell cultures. After 24 hours of incubation, the cells are harvested, the protein solubilized by use of detergents, such as SDS, and the denatured proteins then separated by electrophoresis on a polyacrylamide gel and visualized by autoradiography. The basic methods for HSV-1 are described in detail by other researchers (for example, Spear 1976). In our specific experiments, we used (^{35})methionine as the radiolabel and treated HeLa (a continuous cell line), HEL (human embryonic lung cells that are essentially the same as HESM cells), or COX cells with 1 percent ethanol following infection. The cells were harvested, protein solubilized, and fractionated as described in Anderson, Holland, Gaylord, and Wagner (1980) and Holland et al. (1980). Specific examples of the experiment are shown in figure 5 for HeLa and HEL cells.

Interestingly, host cell protein synthesis in COX cells was not shut off by HSV-1, and although some viral proteins could be visualized, comparisons between ethanol and control cultures were unproductive. In the case of the HeLa and HEL cells, however, it is clear that host cell proteins (seen in the mock infected M-1 bands in figure 5) were efficiently shut off and viral proteins ranging from 155,000 d to ~20,000 d were readily discernible. Ethanol had two effects: (a) one major cellular protein with a size of approximately 50,000 d is less efficiently shut off in the ethanol-treated cultures (open circles, figure 5); and (b) several viral proteins, mainly large ones, are reduced in the ethanol-treated cultures (closed circles, figure 5).

Such results mean that even in cells where low levels of ethanol have little or no effect on virus yield and viral nucleic acid synthesis, expression of specific polypeptides can be altered.

Effect of Ethanol on the Expression of Specific Species of HSV-1 mRNA

In the course of other studies, we have developed powerful techniques for the isolation of HSV-1-specific mRNA and its translation into viral polypeptides (reviewed in Wagner et al. in press). Basically, the method involves preparative hybridization of poly(A) polyribosome-associated mRNA isolated from infected cells. Such hybridization is carried out using either complete HSV-1 DNA or a restriction fragment thereof bound to an insoluble matrix (cellulose). Following hybridization, the cellulose is rinsed exten-

sively to remove nonspecific mRNA and then the purified mRNA can be eluted for further study.

An example of the size distribution of viral mRNA isolatable at the three stages of infection is shown in figure 6. Total HSV-1 polyribosomal poly(A+) RNA, after labeling for 1 hour with (^{3}H)uridine, was isolated from cells at 5 hpi in the absence of protein synthesis and at 2 hpi and 6 hpi under normal conditions of infection (Cx polys). Viral RNA synthesized under these regimes is, respectively, immediate-early RNA, early RNA, and late RNA. Preparative amounts of these three classes of viral mRNA were obtained by hybridizing total poly(A) RNA to HSV-1 DNA bound to cellulose. It is apparent that abundant immediate-early HSV-1 RNA is found only in three size classes: 4.2, 2.8, and 1.8 to 2 kb, as determined from the migration of 28S rRNA and 18S rRNA (5 and 2 kb; McMaster and Carmichael 1977; Wellauer and Dawid 1973). The size distribution of early viral mRNA is considerably more complex, with RNA species ranging from 1.5 to at least 5.2 kb being abundant. The pattern with late viral mRNA is even more complex. The most notable apparent addition is the marked increase in viral mRNA >5 kb in size. These size distributions suggest that the complexity of viral mRNA expression increases with each succeeding stage of viral replication.

The increase in complexity of viral mRNA species with progression of infection can be graphically demonstrated by translation of the viral mRNA isolated from these three stages (see figure 7). Immediate-early RNAs are an efficient template for four major discrete polypeptides of 170,000 d, 120,000 d, 68,000 d, and 64,000 d. Other less prominent ones are also present. Total early viral mRNA is a template for polypeptides of the same size as these. In addition, nine other distinct size polypeptides ranging from 140,000 d to 41,000 d are clearly discernible. Late viral mRNA is a template for in vitro translation of an even more complex population of viral polypeptides: 24 discrete size polypeptides ranging from 155,000 d to 22,000 d or less. It is significant that at least one of the immediate-early polypeptides (170,000 d) is not efficiently translated with viral mRNA from cells at this state of infection. Although the specific numbers of viral polypeptides encoded early and late probably are significant underestimates of the total numbers encoded at the early and late stages of infection, the value of preparative isolation of viral mRNA is readily apparent even with this cursory examination of the complexity of HSV-1 mRNA.

We have described in detail elsewhere how these types of techniques can be used to generally map HSV-1 mRNA (see figure 2 and Wagner et al. in press). In the present case, however, the fact that we can directly translate isolated viral mRNA means that we can ask whether the alterations in viral protein synthesis induced by cocultivation with ethanol are due to direct effects on the expression of HSV-1 genes. We isolated viral mRNA from infected HeLa cells that had been cultured normally for 8 hours after infection or cultured in the presence of 1 percent ethanol and translated 0.5 and 1 μg of such viral mRNA in vitro. A typical autoradiograph of the electrophoretically separated polypeptides synthesized in vitro is shown in figure 8. It is evident that although there is somewhat less overall radioactivity in the ethanol-treated (A) samples compared to the control (E) samples, no specific viral polypeptides were differentially affected by the drug. We conclude, therefore, that the alterations in viral proteins in the infected cell caused by ethanol (see figure 6) are not due to ethanol's effect of viral gene expression per se but rather to alterations in the processing or stability of specific proteins after they are synthesized.

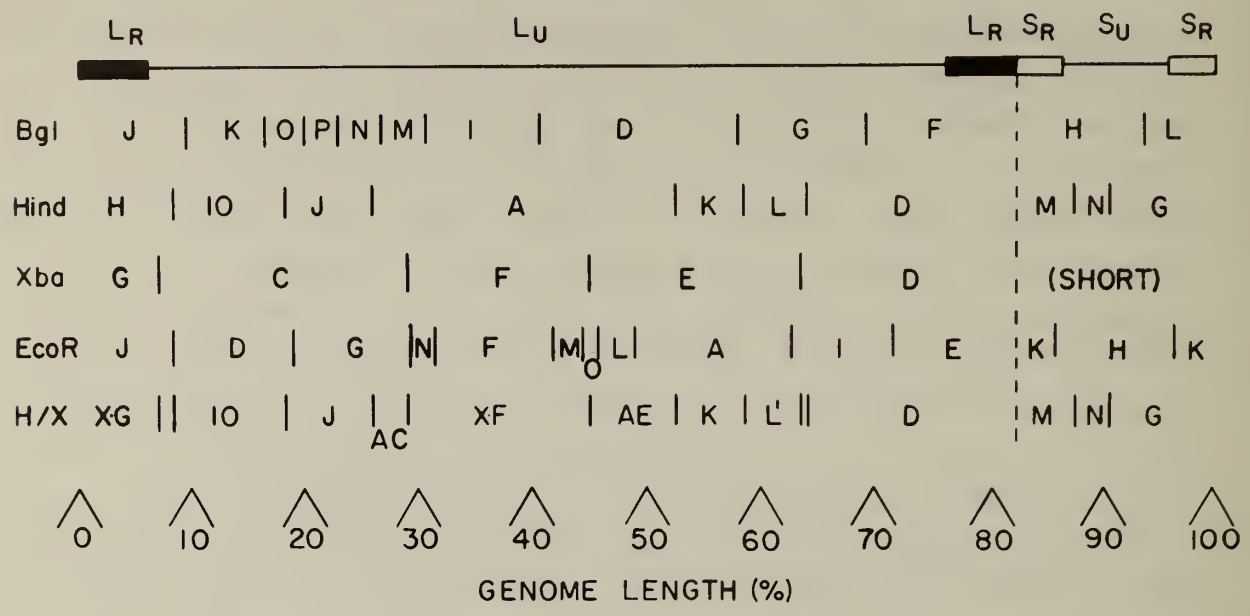
Summary and Conclusions

We have examined the effect of the presence of low levels of ethanol on the processes of HSV-1 infection in model cell cultures. We have established that the virus can replicate in cells of neural origin via different modes, and that in certain cells, ethanol can marginally reduce the yield of infectious virus. We also have found that ethanol has some effect on the level of certain viral proteins. Techniques were developed in our laboratory for the isolation of intact, biologically active HSV-1 mRNA. Thus, we have been able to examine in some detail the types of mRNA and the polypeptides they encode, which become abundant at various stages of the normal infection cycle of the virus and which have specific functions in the life cycle of the virus. We have used these techniques to examine the specific effects of ethanol on HSV-1 gene expression.

Since no particular effects were noted, and since ethanol *does* affect HSV-1 protein metabolism, it must do so at a level beyond primary gene expression. We suggest that insight into these effects

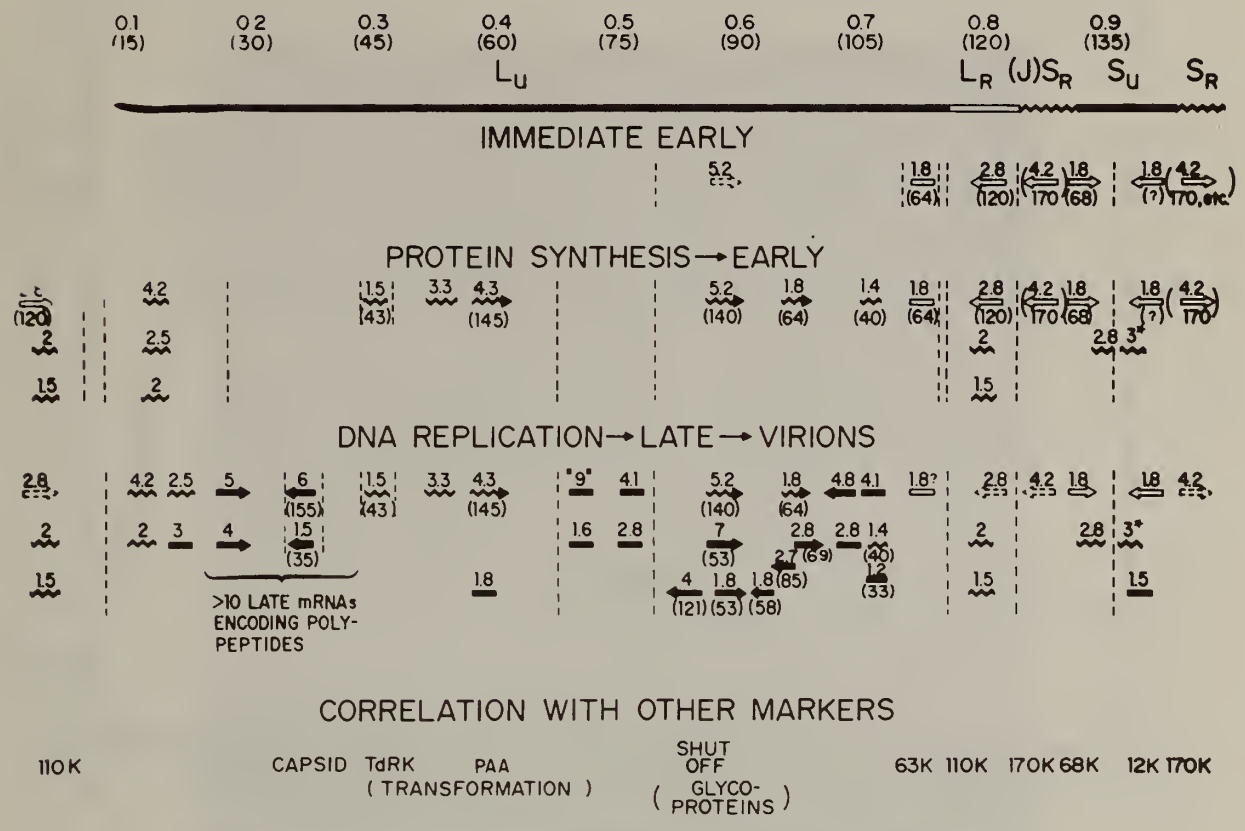
can be attained by investigating specific aspects of viral pathology in animal systems.

Figure 1. Restriction Endonuclease Cleavage Sites for HSV-1 DNA



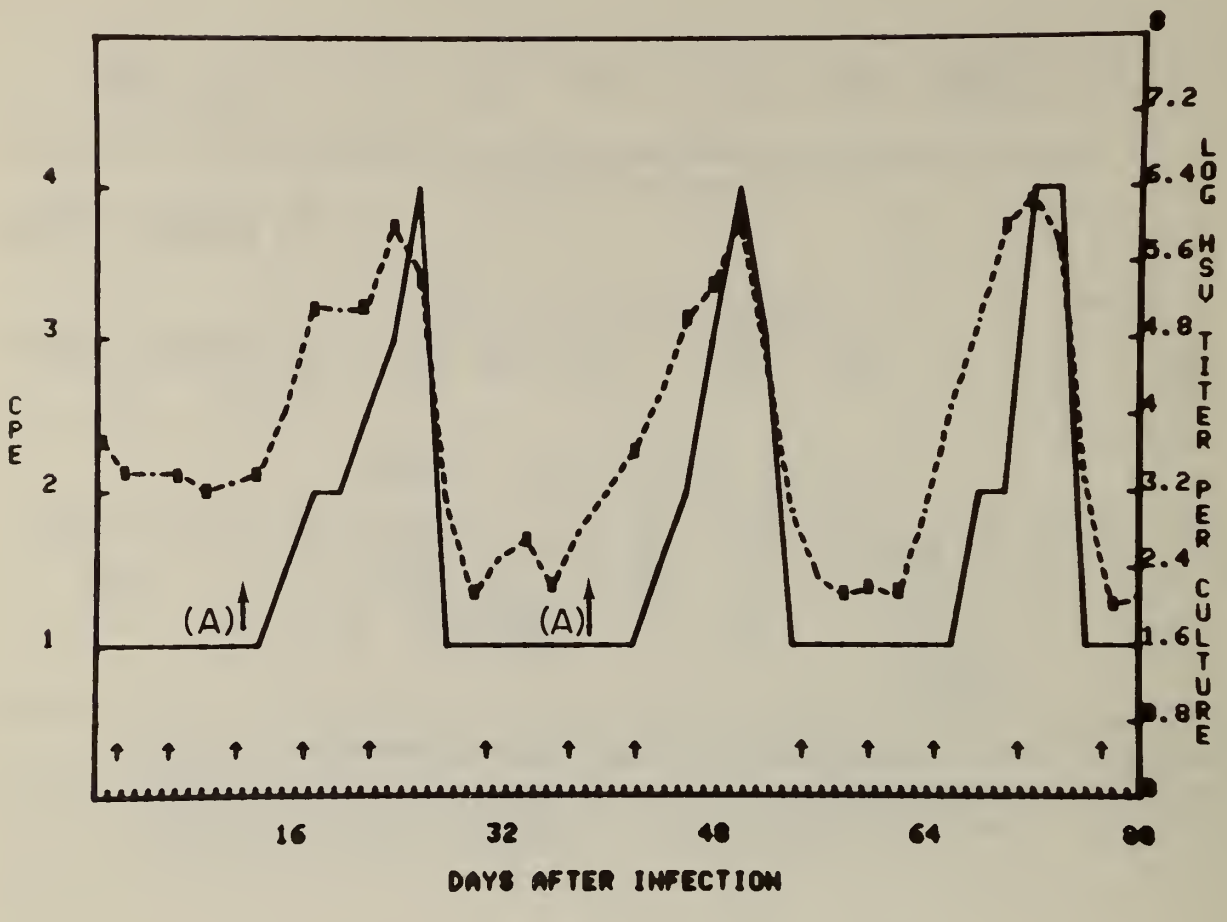
Note: The prototypical (P) arrangement of the HSV-1 genome is shown and restriction cleavage sites for Bgl II, Hind III, Xba I, Hpa I, and Hind III/Xba I double-digests are shown. The whole genome is 150 kb pairs in length. Three other arrangements are possible. These are reviewed by Roizman (1979).

Figure 2. Location of HSV-1 mRNA Species Abundant at the Three Stages of Replication



Note: This figure summarizes studies described in detail elsewhere (Anderson, Costa, Holland, and Wagner 1980; Anderson et al. submitted for publication; Costa et al. submitted for publication; Wagner et al. 1980) and described briefly in this review. Also included are results of experiments currently in progress. Individual mRNA species are localized to the nearest restriction fragment or junction of two fragments found to have significant homology with them. The size of RNA (in kb) is indicated above the location. The direction of transcription, where known, is indicated by arrows pointing toward the 3′ end in the P arrangement of HSV-1. The size of polypeptides encoded (where determined) is shown in thousands of daltons below the mRNA species in question. The locations of other markers were determined from data published by others (Camacho and Spear 1978; Chartrand et al. 1979; Cremer et al. 1978; Maitland and McDougall 1977; Marsden et al 1978; Morse et al. 1978; Powell et al. 1975; Purifoy et al. 1977; Watson et al. 1979; Wigler et al. 1977).

Figure 3. Cytopathic Effect and HSV-1 Yield in Cultures of C-6 Cells



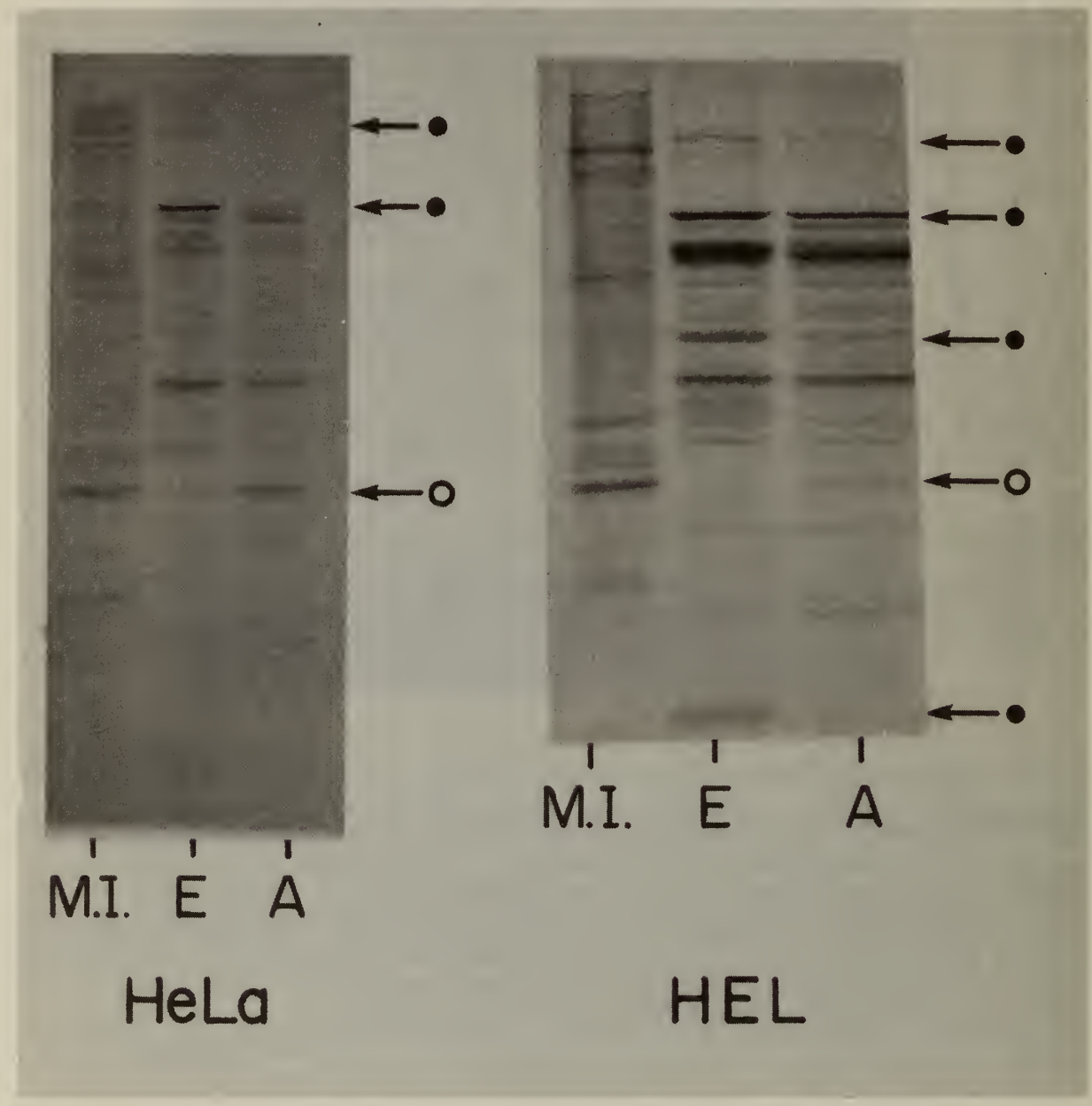
Note: Cells (2 x 10^6) were infected at an MOI of 10 PFU/cell and cultured for the days shown. Cells were passaged at a 1:6 split ratio (arrow) when the density reached 4 x 10^6 cells per flask. Cytopathic effect scale (CPE, solid line): 1, none; 2, barely detectable; 3, obvious cell destruction; 4, great cell destruction, few cells attached to surface. Virus titers (dashed lines) were determined at points marked with the solid rectangles. When cells were subcultured with 0.25 percent ethanol, cytopathology was seen about one passage before that seen in the control cells. This is indicated by the (A ↑) symbols for passages 3 and 7.

Figure 4. Visualization of Unlabeled HSV-1 DNA in EtBr-NaI Gradients

SOURCE: *Archives of Virology,* 59:345-355, 1979. Copyright 1979 by Springer-Verlag, New York, Inc.

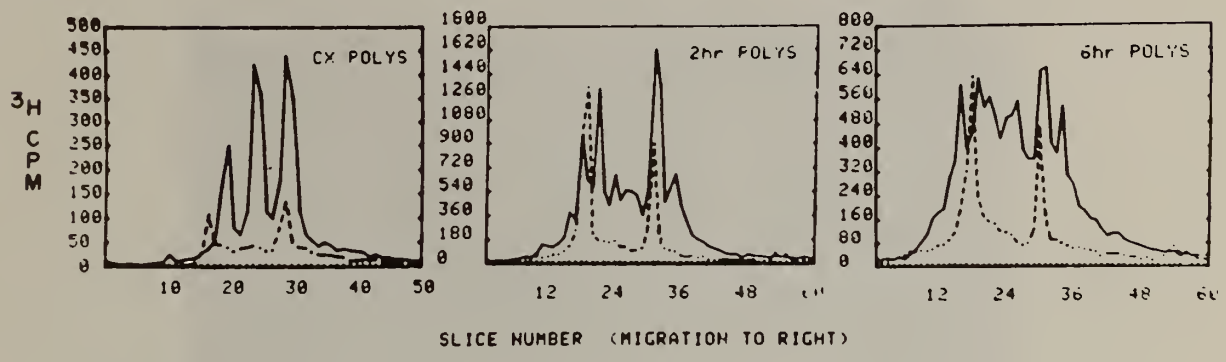
Note: DNA from infected cultures was extracted and fractionated on EtBr-containing NaI gradients. Gradients of infected and control cultures of C-6, D-2, and COX cells are shown. In each case, the broad upper band is cellular DNA; the tight lower band in the COX and D-2 cells is viral DNA (reprinted from Rice et al. 1979).

Figure 5. Influence of Cultivation of HSV-1 Infected Cells With 1 Percent Ethanol on the Synthesis of Viral Proteins



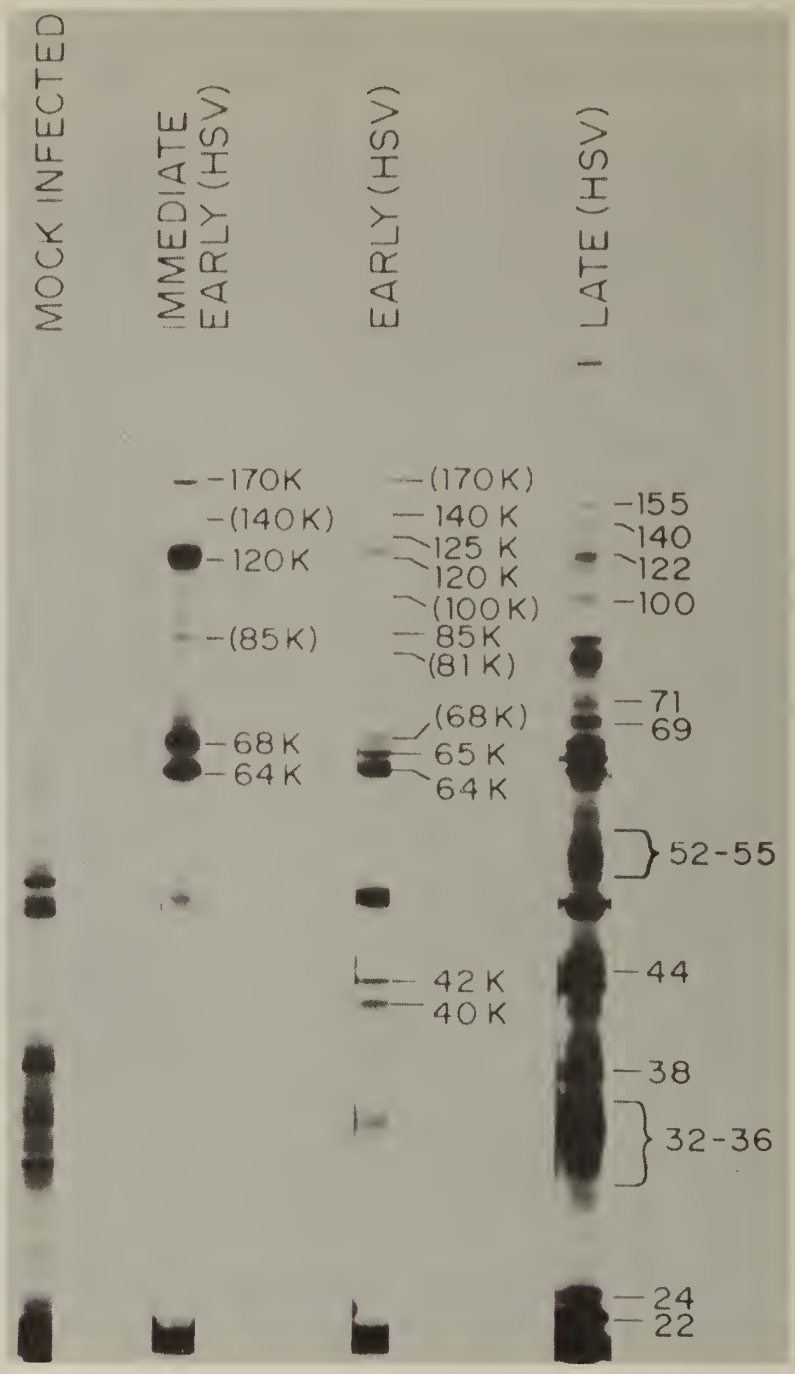
Note: Cultures of 2 x 10^6 of HeLa and HEL cells were either mock-infected (MI) or infected with HSV-1 and incubated from 6 to 24 hpi without (E) or with 1 percent ethanol (A) with (^{35}S) methionine. Cells were harvested and the proteins solubilized by the method described by Spear (1976) and then subjected to SDS-acrylamide gel electrophoresis and autoradiography as described by Anderson, Holland, Gaylord, and Wagner (1980).

Figure 6. Size Distribution of HSV-1 RNA Abundant at the Three Stages of Infection



Note: HeLa cells infected under conditions to yield immediate-early, early, or late RNA were labeled for 1 hour with (^{3}H)uridine. Poly(A+)polyribosomal RNA was isolated as described (Anderson et al. 1979; Holland et al. 1979), and aliquots (4 to 5 μg) were hybridized separately in high formamide to 25 μg HSV-1 DNA bound to cellulose (Anderson et al. 1979). Hybridized RNA was eluted and fractionated on methylmercury agarose gels (Bailey and Davidson 1976). The dashed lines are the positions of ^{32}P-labeled 28S and 18S rRNA included as a marker.

Figure 7. In Vitro Translation Products of Immediate-Early, Early, and Late HSV-I mRNA



Note: Aliquots (0.3 to 0.6 μg) of HSV-1 poly(A+) mRNA isolated from the three stages of infection were purified by hybridization to DNA cellulose (as described in figure 6), translated in a commercial reticulocyte system (Anderson et al. 1979; Anderson, Holland, Gaylord, and Wagner 1980; Holland et al. 1979), and fractionated on 9 percent polyacrylamide-SDS gels (Laemmli 1970). Sizes of polypeptides, indicated in thousands of daltons, were determined from the migration of adenovirus markers. The heavy band of material migrating at 50,000 d is endogenous to the system.

Figure 8. Influence of Cultivation of HSV-1 Infected Cells With 1 Percent Ethanol on the Information Content of Viral mRNA

Note: HSV-1 infected HeLa cells were incubated for 8 hours in the absence of (E) or the presence of (A) 1 percent ethanol. Polyribosomal poly(A) mRNA was isolated and viral mRNA purified as described by Holland et al. (1980). This mRNA was then used in a commercial reticulocyte lysate system for the in vitro translation of HSV-1 polypeptides as described in Anderson, Holland, Gaylord, and Wagner (1980). Also shown are a no-RNA and an adenovirus mRNA control.

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Table 1. Yield of HSV-1 in Neural Cells[a]

Cells and Origin	PFU/Cell	
	24 hpi[b]	24 hpi in the presence of 1.0 percent ethanol[c]
HESM, human embryo	65 ± 10	50 ± 10-
D-2, mouse neuroblastoma	25 ± 5	not done
COX, human astrocytoma	30 ± 7	18 ± 7
C-6 rat glioma	0.01 ± 0.005	0

[a] Infections were carried out at an MOI of 5 PFU/cell.

[b] Data from three experiments.

[c] Data from two experiments.

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The Effects of Ethanol and Acetaldehyde on Brain Protein and DNA Synthesis Are Independent of Nutritional Effects*

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Abstract

We studied brain protein synthesis in vivo by the method of Dunlop et al. (1975) in brain slices and in cell-free systems in vitro. In rats chronically addicted to ethanol, but not in paired naive controls, moderate ethanol loads resulted in a highly significant inhibition of brain protein synthesis in all regions studied. This inhibition was independent of the nutritional state of the animal, as demonstrated by use of pair-fed controls and paired weight controls. The inhibition was reversible upon discontinuation of the treatment. In immature animals, profound inhibition of protein synthesis and even greater inhibition of DNA synthesis were induced by both ethanol and acetaldehyde, the latter being effective at much lower concentrations. These results confirmed the findings of other investigators and will be discussed in relation to the cerebral atrophy observed in the vast majority of alcoholics by CAT scan and to the possible occurrence of fetal alcohol syndromes in humans.

NOTE: Figures and table appear at end of paper.

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Introduction

In recent years, it has become clear that chronic ethanol ingestion can lead to severe and progressive liver disease even in the absence of malnutrition (Lieber et al. 1975). The earliest manifestations of the liver's response to ethanol include an inhibition of albumin synthesis and a dissociation of membrane-bound polyribosomes. Several studies have suggested that the brain may be similarly affected and eventually damaged, and recent investigations have shown brain atrophy (figure 1) in a large number of human chronic ethanol abusers (Carlen et al. 1978; Newman 1978). Several studies have demonstrated inhibition of brain protein synthesis during chronic ethanol ingestion in animal models (Fleming et al. 1975; Jarlstedt 1972; Kuriyama et al. 1971; Tewari and Noble 1971). However, none of these studies used the improved in vivo methods developed by Dunlop et al. (1975) in a model with strict nutritional controls. The present study fulfills these criteria and demonstrates that in rats the chronic (but not the acute) ingestion of moderate amounts of ethanol results in a chronic inhibition of brain protein synthesis compared to pair-fed and weight-matched controls (Lieber and DeCarli 1973).

Materials and Methods

In experiment 1, male Sprague-Dawley rat littermates matched for weight were fed either a liquid diet containing 6 percent ethanol (Lieber and DeCarli 1973) or a similar complete diet except for the isocaloric replacement of ethanol by carbohydrates.

The chronic ethanol group received the experimental diet for 1 month. To avoid variations due to differences in feeding or drinking patterns, the animals were fed by nasogastric tube between 7 a.m. and 9 a.m. on the day of the experiment a quantity of diet equivalent to their average daily intake. Controls received the same amount of control diet by gavage. Acute ethanol animals were untreated except on the day of the experiment, when they received the same amount of ethanol-containing diet by gavage as did the chronic ethanol group. Measurements of protein synthesis were made between 10 a.m. and 12 noon on the same day. However, one group of chronically addicted animals was studied between 3 p.m. and 6 p.m. after a 7 a.m. to 8 a.m. gavage. By 3 p.m., withdrawal

seizures had occurred and abated in the majority of experimental animals.

In experiment 2, each experimental animal was paired with two controls. A pair-fed control (PC) received the same amount of diet as the enthanol-addicted rat (E). The weight control (WC) received smaller amounts of the control diet than the pair-fed control in order to keep its weight at or below the level of its ethanol-addicted littermate.

In Vivo Measurements of Brain Protein Synthesis

(^{3}H)lysine (specific activity greater than 20 Ci/mmol) and (^{14}C)valine (specific activity 100 mc/mmol) were diluted with cold amino acids to a final activity of 0.1 μCi/μmol. These methods have been described in detail by Wasterlain and Fando (1980). Animals were injected intraperitoneally with a solution containing (^{3}H)lysine (0.1 μCi/μmol; 10 μmol/g) and (^{14}C)valine (0.1 μCi/μmol; 10 μmolg) between 10 a.m. and 12 noon at the onset of the experiment. Valine was omitted in experiment 2. The animals were decapitated after 2 hours. Preliminary experiments demonstrated that incorporation of amino acids was linear over the 2 hours of the experiment. To ascertain that the larger amounts of amino acids injected did not alter the rate of brain protein synthesis, a group of untreated rats received intraperitoneal injections of the same amino acid solution in amounts varying from 2 to 14 nmol/kg. They were sacrificed after 2 hours and processed as described below. The brain and other organs were placed in individual vials, weighed, and quickly frozen in liquid nitrogen. Forebrain (defined as the part of the brain located below the olfactory lobes and above the inferior colliculi), brainstem (located between the intercollicular cut and the obex of the fourth ventricle), and cerebellum (separated from brain stem by a horizontal cut through the cerebellar peduncles) were dissected free hand and homogenized in 10 volumes of 10 percent trichloracetic acid (TCA). The radioactivity and specific activity of valine and lysine in the acid-soluble supernatant were measured. The precipitate was washed twice in 5 percent TCA, once in 100 percent methanol, and twice in methanol-chloroform (1:2). The final pellet was thoroughly dried, then dissolved in 1 N NaOH. Aliquots were used for measurements of protein concentration (Lowry et al. 1951) and radioactivity (after neutralization).

Protein and DNA Synthesis in Brain Slices

Slices of forebrain cortex from immature animals were incubated in the presence of labeled amino acid or labeled thymidine using previously described methods (Dunlop et al. 1974; Wasterlain 1976).

Results

Clinical Observations

During their first few days on the diet, the chronic ethanol animals frequently appeared dazed and ataxic. However, within a week to 10 days both coordination and alertness returned to a level similar to that of controls. Within a few hours of the last ethanol injection, chronic ethanol animals developed excitation, piloerection, motor hyperactivity, increased reaction to external stimuli, and, in the majority of animals, major motor seizures. None of these signs was observed in the acute ethanol group.

Effects of Amino Acid Loads on Brain Protein Synthesis

Intraperitoneal injection of amino acid loads ranging from 2 to 30 nmol/kg of each amino acid did not alter the rate of protein synthesis in forebrain (figure 2). Loads of 40 nmol/kg resulted in a severe inhibition of both valine and lysine incorporation into brain proteins. These results confirm those of Dunlop et al. (1975) and suggest that the amounts of amino acid used in the study (10 mmol/kg) did not interfere with our measurements of the rate of brain protein synthesis.

Effects of Ethanol

In forebrain, the rate of lysine incorporation into proteins was similar in acute control animals (2.09 $\pm$ 0.03 nmol/mg of protein per hour), in chronic controls (2.09 $\pm$ 0.07), and in acute ethanol rats (2.04 $\pm$ 0.12, -2.5 percent NS) (figure 3). Animals fed ethanol for 1 month had a significantly lower rate of lysine incorporation into proteins than did the other three groups (1.62 $\pm$ 0.07 nmol/mg of protein per hour, $p < .01$). This 22.6 percent reduction in the rate

of amino acid incorporation was not accompanied by any change in the specific activity of the free lysine pool, which was not significantly different in any of the four groups studied (figure 3). This result suggests that the rate of lysine incorporation in this experiment accurately reflected the rate of protein synthesis in forebrain. The rate of lysine incorporation into cerebellum and brain stem was similarly lower in the chronic ethanol group than in the other three groups. The rate of valine incorporation was measured only in chronic animals. This rate was significantly reduced by ethanol intake compared to pair-fed controls in all three brain regions studied. Baseline values for valine incorporation into brain proteins were slightly higher than those of lysine incorporation, and the degree of inhibition was slightly less (-13.6 percent versus -22.6 percent in forebrain, -11.7 percent versus -20 percent in cerebellum, and -19.5 percent versus -18.3 percent in brain stem) (figure 4).

The slight, nonsignificant trend toward lower values in cerebellum and brain stem in chronic controls compared to acute controls might reflect experimental error or possibly that the food intake of "chronic control" rats was restricted so that they would not eat more than their paired ethanol-addicted littermates. Lysine incorporation was measured in a separate group of animals whose withdrawal seizures and agitation had abated and in their pair-fed controls. Standard errors were larger than in the group described in figure 3, and no differences were observed between experimental and controls (forebrain, C=1.76 $\pm$ 0.24 nmol/mg of protein per hour; E=2.20 $\pm$ 0.24, NS; cerebellum C=1.60 $\pm$ 0.19, E=1.64 $\pm$ 0.19, NS; brain stem, C=1.54 $\pm$ 0.15, E=1.58 $\pm$ 0.11, NS; muscle, C=0.93 $\pm$ 0.14, E=1.14 $\pm$ 0.13, NS). It would thus appear that the reduction of brain protein sythesis in chronic ethanol animals disappears rapidly upon withdrawal.

In both chronic groups, lysine and valine incorporation were compared in heart muscle, skeletal muscle, and liver (figure 5). No significant differences were observed following the moderate amounts of ethanol administered. However, a small trend toward reduction of synthetic rates was noted in skeletal muscle. The ratio of valine to lysine incorporated varied from 0.94 in chronic control animals to 1.48 in chronic ethanol animals, suggesting a change in composition of proteins synthesized. Measurements of the rate of lysine and valine incorporation into lung, kidney, and spleen (figure 6) revealed no significant differences.

Nutritional Controls

Weight gain was greater in the pair-fed control group than in the ethanol-addicted rats, whereas weight controls were effectively kept at a weight close to that of the experimental littermates. The rate of lysine incorporation into brain proteins was similar in both control groups and was lower in the ethanol-addicted group than in either control, suggesting that the greater body weight gain of pair-fed animals had no effect on their rate of brain protein synthesis. These results suggest that even in the complete absence of malnutrition, chronic ethanol addiction inhibits brain protein synthesis. The changes were observed both in vivo and in cell-free systems in vitro (table 1). In view of the lack of change in precursor activity for (^{3}H)lysine and of previous studies using double label, the rate of lysine incorporation was probably a good reflection of the rate of brain protein synthesis. Additional confirmation of that conclusion was obtained in vitro. No cerebral atrophy was observed in these animals, but the duration or intensity of ethanol exposure may have been too short to alter brain weight.

Effects of Ethanol and Acetaldehyde on Brain Slices

One puzzling aspect of our findings in brain was the difference between acute and chronic ethanol animals, all of which had received similar amounts of ethanol on the day of the experiment. One possible explanation of the difference might relate to the difference in ethanol or acetaldehyde levels as a function of chronic exposure to ethanol. Korsten et al. (1975) reported that animals and men chronically addicted to ethanol had higher blood acetaldehyde levels than did controls after a similar ethanol load. Raskin and Sokoloff (1974) showed induction of brain alcohol dehydrogenase by ethanol, suggesting that chronic ingestion may increase acetaldehyde formation. Therefore, we studied the effects of ethanol and acetaldehyde on protein synthesis (figure 7) and DNA synthesis (figure 8) in brain slices. In this experiment, we used 4-day-old rats, since inhibition of protein synthesis would have its greatest significance in the developing brain. Ethanol produced only small and inconsistent inhibition of lysine incorporation into brain slice proteins, but acetaldehyde at concentrations of 0.2 mM or higher profoundly inhibited protein synthesis in a dose-dependent fashion (figure 7). Thymidine incorporation, probably reflecting DNA

synthesis, also showed little change as a function of ethanol concentration (figure 8), whereas acetaldehyde severely inhibited DNA synthesis. The concentration of acetaldehyde reached in rat blood after acute injection of moderate doses of ethanol (3 g/kg) are in the 0.2 to 0.4 mM range, at which significant inhibition of DNA and protein synthesis was observed. Although a similar blood level of acetaldehyde was found in mouse following ethanol administration (3 g/kg intraperitoneally) substantially lower levels were found in brain tissue (Tabakoff et al. 1976). Thus the precise role of acetaldehyde in the gross inhibition of protein and DNA synthesis is uncertain.

Discussion

These findings confirm and extend the results of previous investigators who observed an inhibition of brain protein synthesis and of brain growth by ethanol (Fleming et al. 1975; Jarlstedt 1972; Kuriyama et al. 1971; Tewari and Noble (1971). The measurement of brain protein synthesis during chronic ethanol treatment is particularly difficult because of the effect of ethanol on brain amino acid metabolism and the problems in assessing the exact effect of possible changes in subcellular amino acid pools on the apparent rate of synthesis. In this study, several methods were used to circumvent these problems. First, the use of the Dunlop-Lajtha method, with injection of large amounts of amino acid precursors, floods all subcellular pools and makes the measurements independent of variations in pool concentrations, since all pools are likely to have similar specific activities. In our studies, the independence of the measured rate of synthesis from amino acid concentrations was directly verified and precursor specific activity was directly measured. Second, the use of a double-label method, in which one amino acid is basic and a second amino acid (in this case, (1-^{14}C)valine) is neutral and uses a different carrier across the blood-brain barrier, permits a direct verification that the measured rate of protein synthesis is independent of amino acid transport. These results suggest that the observed changes in amino acid incorporation actually reflected changes in the rate of brain protein synthesis. Third, in vivo measurements were coupled with in vitro measurements both in the brain slices and in cell-free systems. Cell-free systems are not subject to errors due to varying amino acid

specific activities in different subcellular pools. Studies in brain slices are immune to changes in systemic or blood brain barrier transport or metabolism, and in vivo measurements are obtained in a physiological setting, so that the conclusions confirm each other and the results of previous investigators.

The inhibition of brain protein synthesis in ethanol-addicted animals was independent of malnutrition. It has long been established that chronic ethanol intake inhibits brain protein synthesis and that this inhibition is observed even in the presence of relatively good nutrition in the ethanol-addicted animals (Tewari and Noble 1971). In the current investigation, the use of paired feeding with Lieber-DeCarli diet demonstrated that differences in food intake were not responsible for the observed differences in brain protein synthesis but were insufficient to completely rule out the role of malnutrition, since the ethanol-treated animals gained less weight than their pair-fed controls. However, weight-matched animals fed a similar diet in lesser amounts than the ethanol-treated group had rates of amino acid incorporation in brain that were similar to controls and higher than those of ethanol-addicted animals.

The current findings may be relevant to the cerebral atrophy observed by CAT scanning in nearly all humans addicted to ethanol (Newman 1978), a condition that may be reversible upon discontinuation of alcohol intake (Carlen et al. 1978). Cerebral atrophy may also be at the source of some of the dendritic changes observed in the hippocampus during ethanol addiction (Riley and Walker 1978). It is interesting in this respect that animals chronically addicted to ethanol demonstrated a significant inhibition of brain protein synthesis after ethanol loads that had no significant effects on the brains of naive animals. Several previous studies have shown that for the same ethanol load, acetaldehyde concentrations in blood are increased in chronic alcoholics compared to controls (Korsten et al. 1975) and that the susceptibility to acetaldehyde may also be increased (Matsuzaki and Lieber 1977). The lack of effect of ethanol on cell cultures except in very high concentrations (Seil et al. 1977) and the profound inhibition of protein synthesis in cardiac muscle (Rawat 1979) by acetaldehyde rather than ethanol might also suggest that the greater inhibition of brain protein synthesis in the chronic animals may have reflected an acetaldehyde effect. In our slice experiments, both protein and DNA synthesis were more sensitive to acetaldehyde than to ethanol, and DNA synthesis was inhibited at lower concentrations than was protein synthesis. This

result might suggest a mechanism for the effect of ethanol on protein synthesis in the ill-defined fetal alcohol syndrome and in the effects of ethanol on brain growth. Since 80 percent of all brain cells are generated postnatally, and DNA synthesis is very active in the first 2 years of life, these experiments further emphasize the well-known dangers of ethanol for the immature brain. However, it is doubtful that the concentrations needed to inhibit protein synthesis in brain slices are reached in brain during ethanol abuse, so the significance of these effects will require further investigation.

Mechanism of Polysomal Dissociation by Ethanol: A Hypothesis

We recently demonstrated that increases in GDP/GTP ratios have a profound inhibitory effect on ternary complex formation, the first step of initiation of translation (Dwyer and Wasterlain 1980). This presumably acts as an amplification system that permits the production in brain of a large signal for a small decrease in GTP, so that protein synthesis initiation is shut off before GTP levels fall enough to compromise cell survival. This inhibition of ternary complex formation may be responsible for the dissociation of brain polysomes following seizures (Vesco and Giduitta 1968; Wasterlain 1977) or partial ischemia (Cooper et al. 1977; Kleihues and Hossman 1971) by blocking initiation in response to minimal changes in cerebral energy charge potential, too small to affect other steps of the protein synthetic mechanism, so that elongation proceeds in the absence of initiation, resulting in "run-off" ribosomes and subunits that associate into inactive 115s pseudodisomes (Wasterlain 1977). We speculate that a similar mechanism may be responsible for the main polysomal dissociation induced by ethanol. This hypothesis is supported by the observed fall in brain ATP following ethanol (Rawat 1973) and by the high activity of nucleotide diphosphate kinase in brain, so that decreases in brain ATP are reflected in a cascade of decrease in brain GTP and rise in brain GDP (Atkinson 1968).

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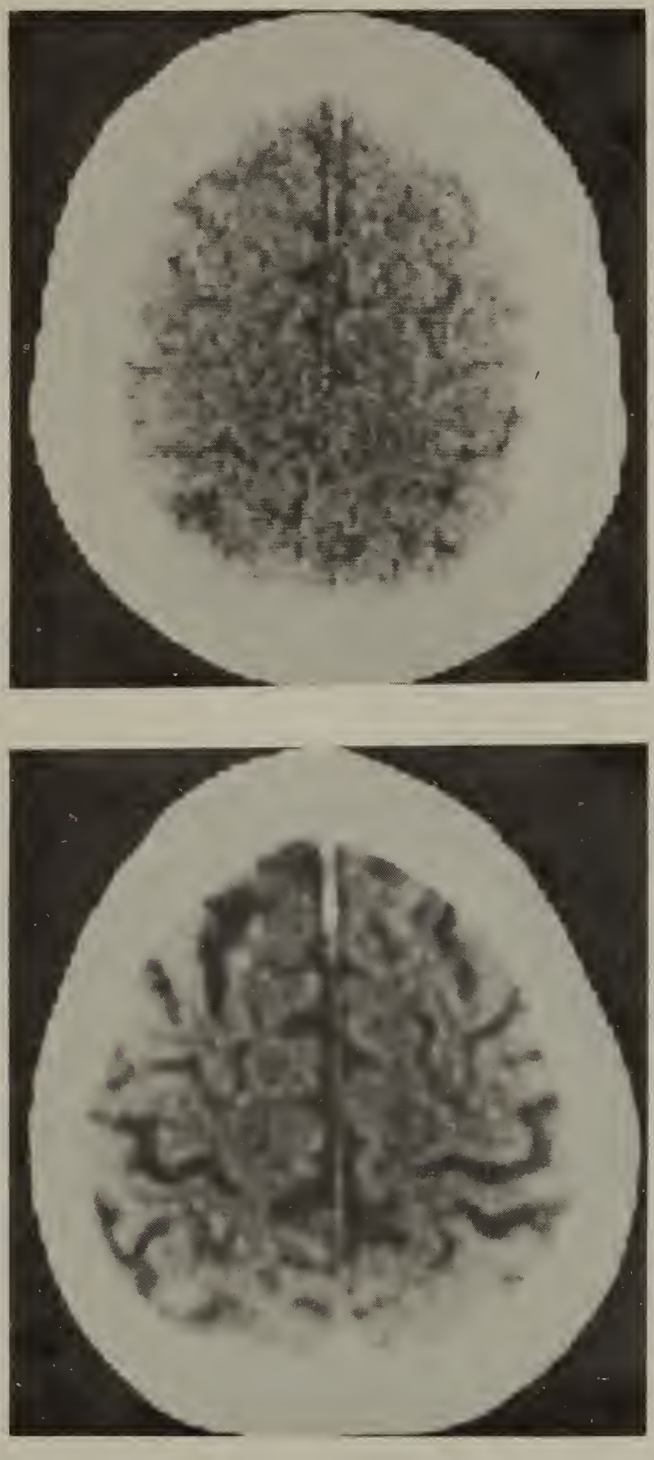
Conclusions

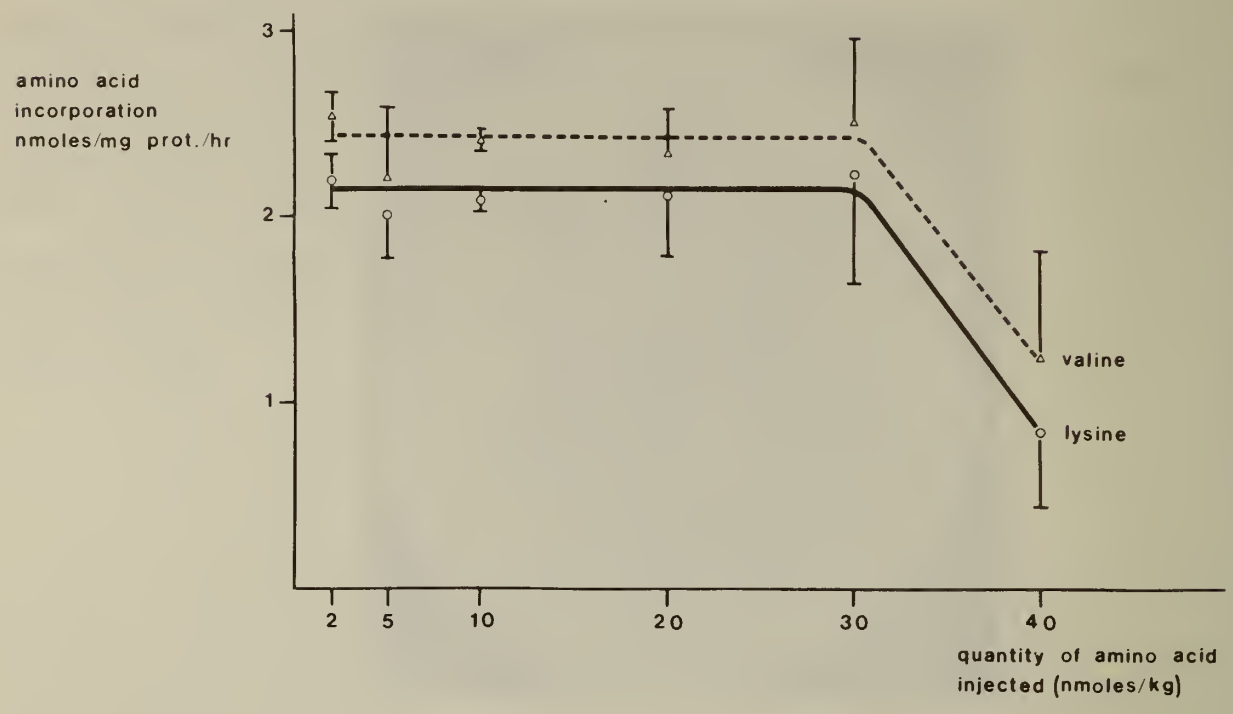
Our data confirm the findings of other investigators that chronic ethanol addiction inhibits brain protein synthesis and that this inhibition is not simply a result of malnutrition. The data also raise the possibility of a role of acetaldehyde in these ethanol actions. Understanding the relationship between the effects of ethanol or brain protein and DNA synthesis and the consequences of chronic alcoholism in humans, such as cerebral atrophy or the fetal alcohol syndrome, may be one of our major scientific challenges for the years ahead.

Figure 1. Computerized Tomographic Images of the Brain of a 57-Year-Old Male With No Evidence of Brain Disease (A) and of a 57-Year-Old Male Chronic Alcoholic (B)



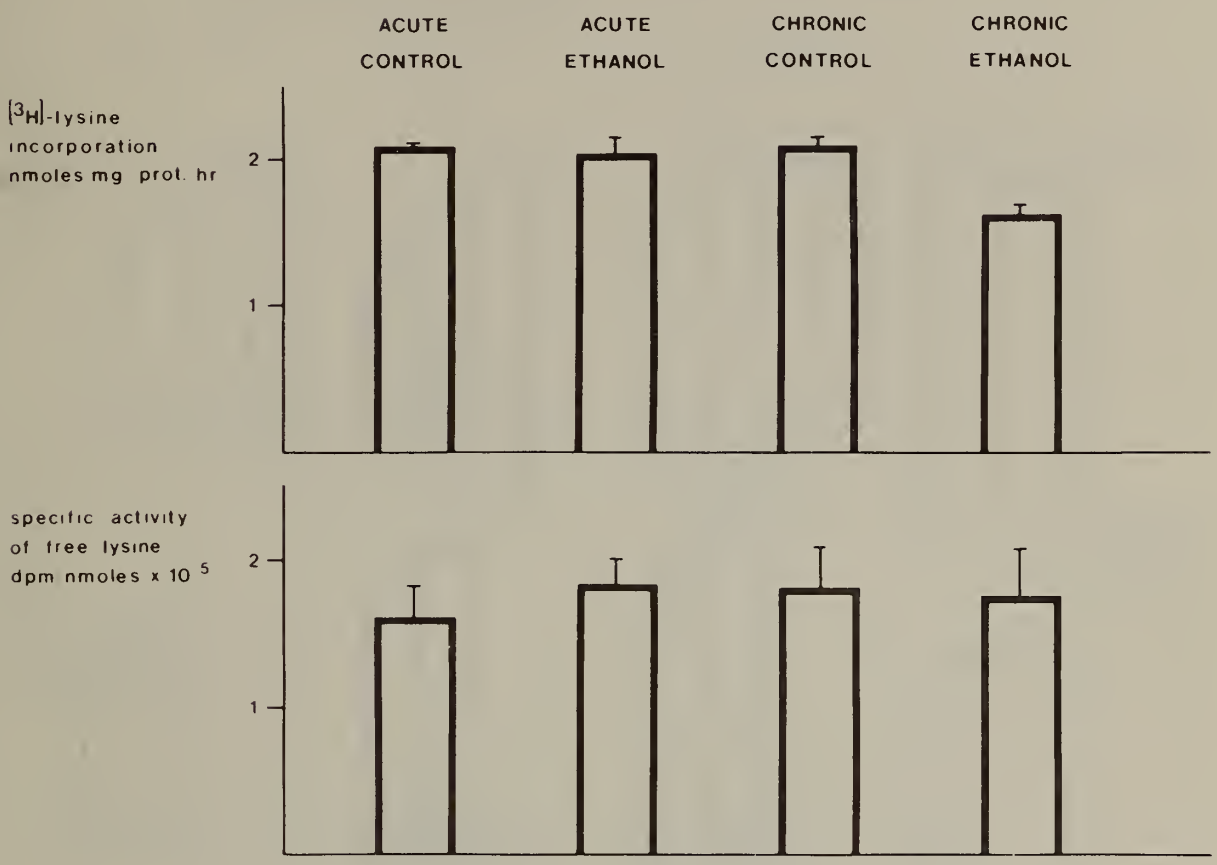
Note: These scans show the cortex in the upper part of both hemispheres. The (dark) sulci are clearly enlarged in B, reflecting the cerebral atrophy observed in the vast majority of chronic alcoholics.

Figure 2. Effect of Amino Acid Load on Brain Protein Synthesis



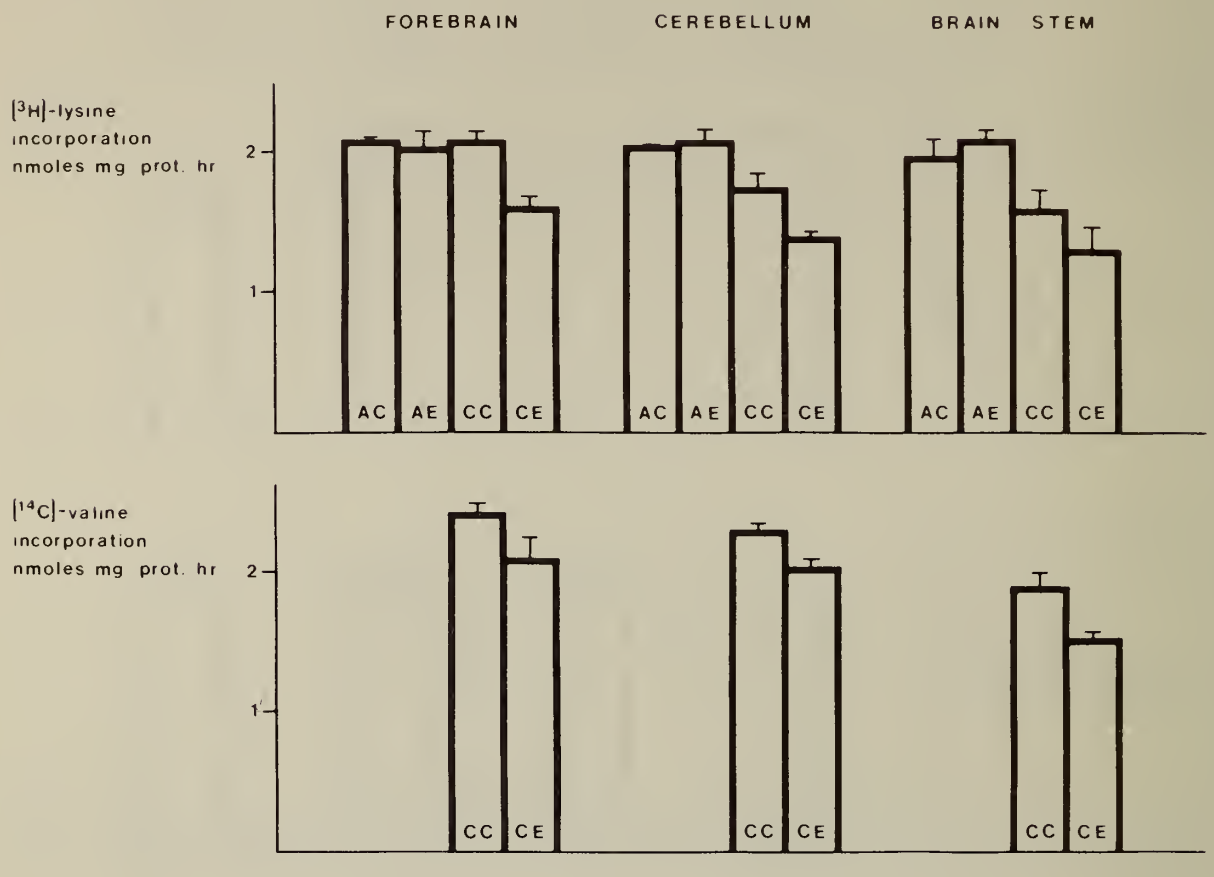
Note: Rats were injected with (^{3}H)lysine (0.1 μCi/μmole) and (1-^{14}C)valine (0.1 μCi/μmole) over the range of doses shown. Rats were sacrificed after 2 hours, and the brains were homogenized in 10 percent trichloroacetic acid. The acid-precipitable material was delipidated and dissolved in 1N NaOH for determination of protein and radioactivity. Incorporation of lysine and valine is expressed as nanomoles of amino acid incorporated/mg/protein/hour.

Figure 3. Effects of Ethanol on Forebrain Protein Synthesis



Note: Rats were fed Lieber-DeCarli diet containing ethanol or isocaloric carbohydrate for 1 month (chronic group). On the day of the experiment, a loading dose of ethanol-containing diet equal to the average daily intake of the chronic rats was given to both chronic and acute ethanol rats between 7 a.m. and 9 a.m. Protein synthesis was measured between 10 a.m. and 12 noon. Rats were injected with (^{3}H)lysine (10 μmol/g; 0.1 μCi/μmol) and sacrificed 2 hours later. Rates of amino acid incorporation were determined as described in figure 2. Amino acid specific activity was measured in the acid-soluble fraction from brain.

Figure 4. Effects of Ethanol on Protein Synthesis in Brain Regions



Note: Experimental conditions were as described in figure 3. (^{3}H)lysine (0.1 μCi/μmol/g), and in addition in chronic animals (1-^{14}C)valine (0.1 μCi/μmol/g), was injected and animals were sacrificed 2 hours later.

Figure 5. Effects of Ethanol on Amino Acid Incorporation Into Proteins of Heart Muscle, Gastrocnemius Muscle, and Liver of Ethanol-Treated Rats and Controls

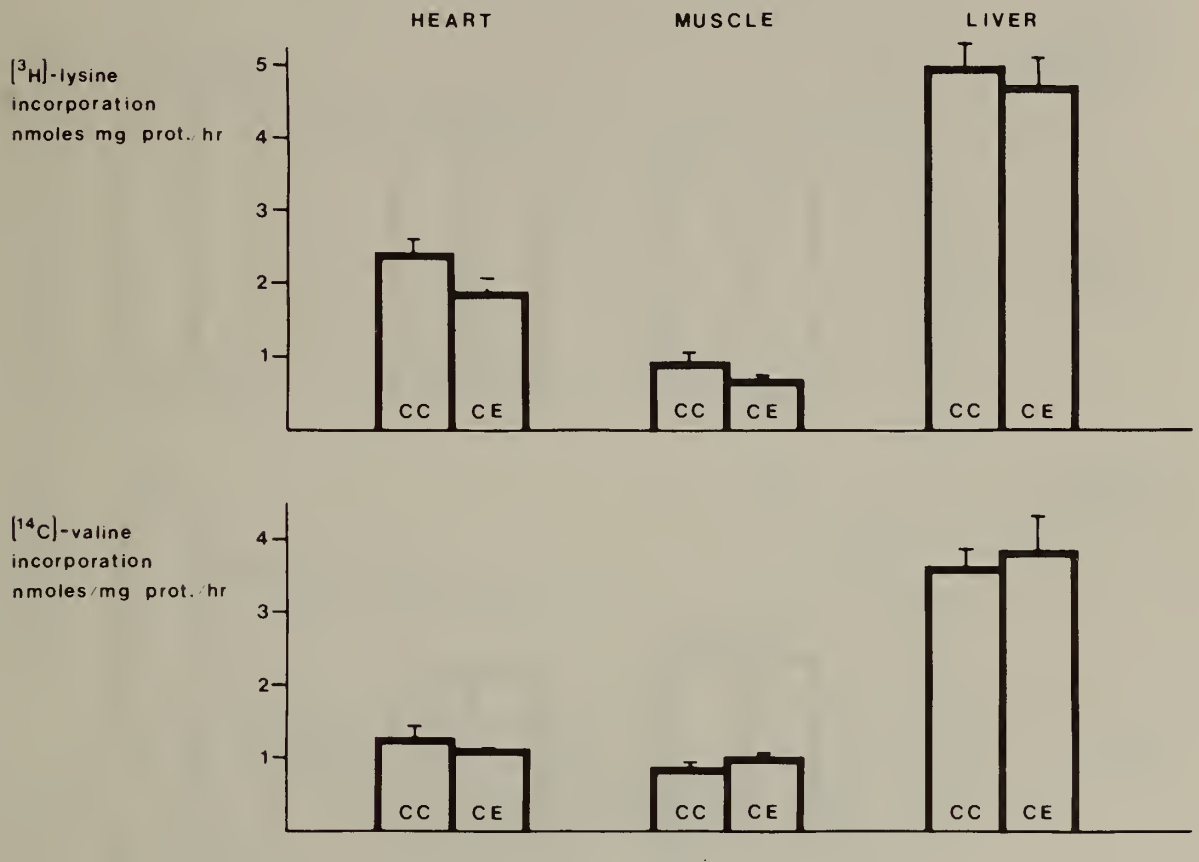


Figure 6. Effects of Ethanol on Amino Acid Incorporation Into Proteins of Lung, Kidney, and Spleen of Ethanol-Treated Rats and Controls

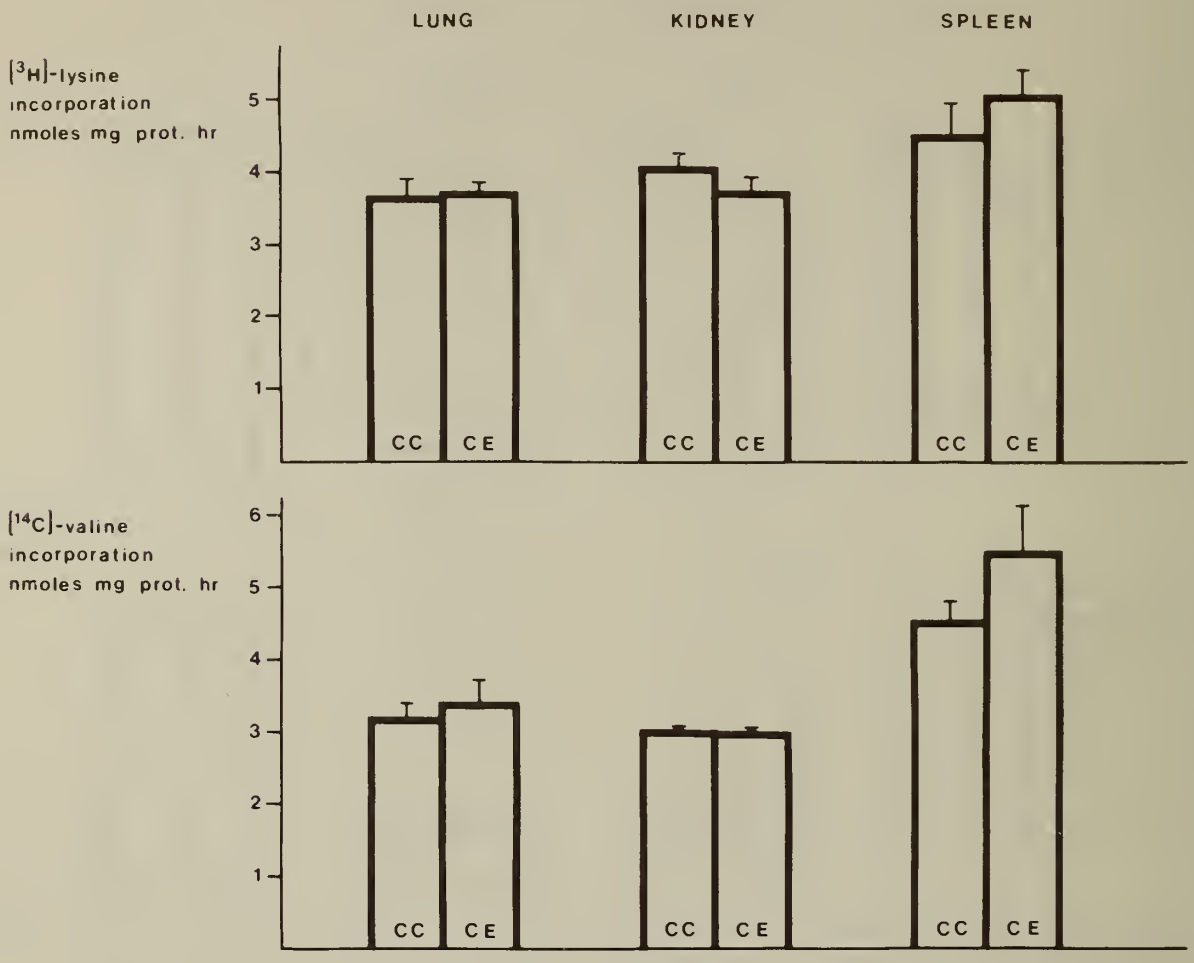
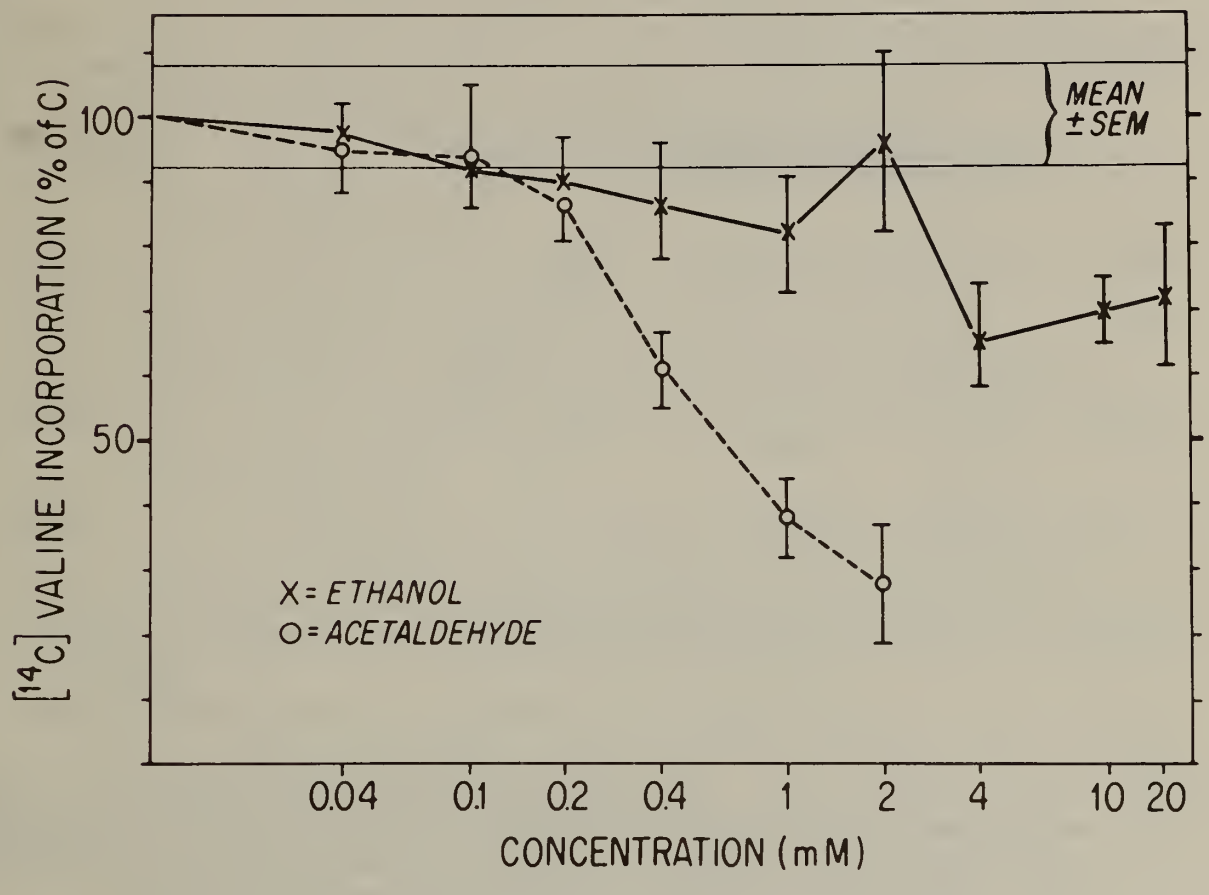
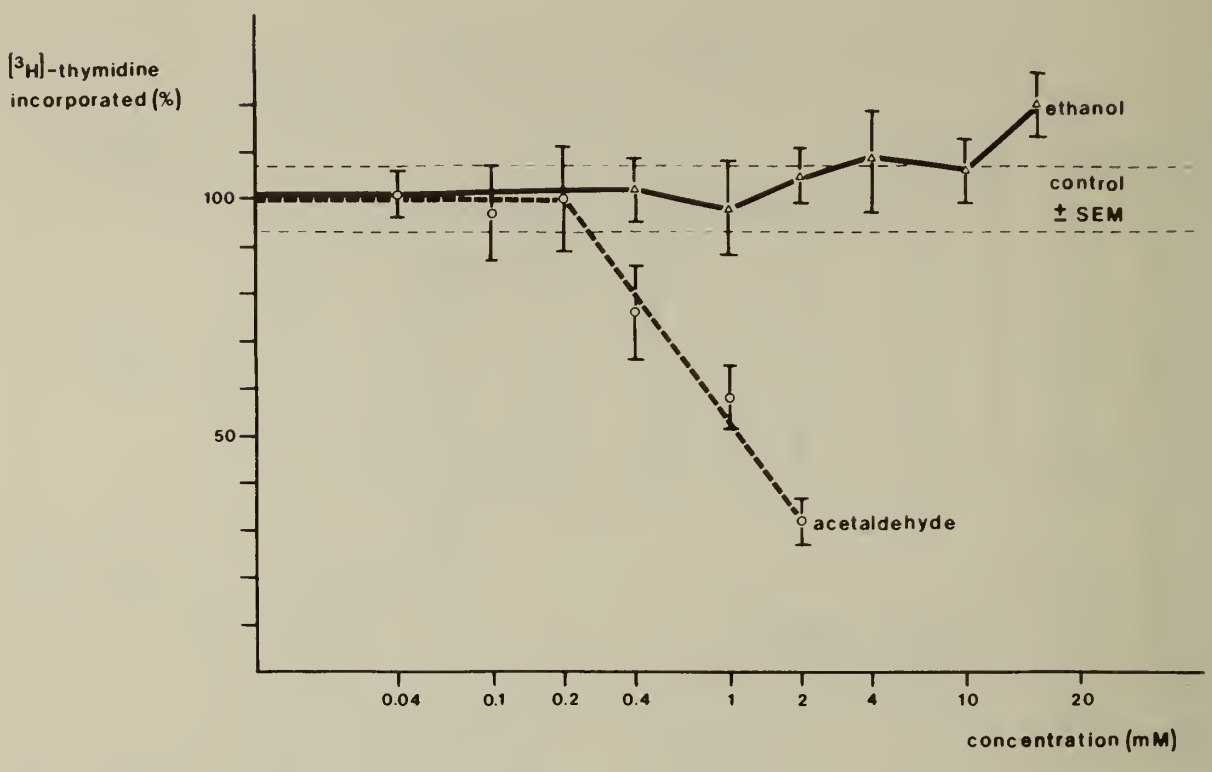


Figure 7. Effects of Ethanol and Acetaldehyde on Protein Synthesis in Brain Slices



Note: Slices obtained from cerebral cortex of 4-day-old rats were preincubated for 1 hour in Krebs-Ringer solution with glucose under a constant flow of 95 percent O_2 and 5 percent CO_2. They were then treated with ethanol or acetaldehyde, and with (^{14}C)valine (0.1 mM, sp. Act. 0.1 mCi/mmol). Data are expressed as percentage of controls ± SEM.

Figure 8. Effects of Ethanol and Acetaldehyde on DNA Synthesis in Brain Slices



Note: Slices obtained from the cerebral cortex of 4-day-old rats were preincubated for 1 hour as described in figure 7. They were treated with (^{3}H)thymidine (2 μCi/2 ml, Sp Act. 84 Ci/mmol) instead of valine. Data are expressed as percentage of controls ± SEM.

Table 1. In Vivo and In Vitro Changes in Brain Protein Synthesis

	Ethanol-Addicted	Pair-Fed Controls	Weight-Matched Controls
In Vivo (^{3}H)Lysine Incorporation (nmol/mg protein/hr)	1.72 ± 0.06	2.11 ± 0.08	2.07 ± 0.04
In Vitro (^{3}H)Lysine Incorporation (CPM/mg protein/hr)	11695 ± 1907	15948 ± 376	15692 ± 972

Note: Ethanol rats were adult male Spague-Dawleys and received liquid (Lieber-Decarli) diet for 1 month. Both control groups were paired littermates of the experimental rats of similar body weight (± 5 g) at the onset of the experiment. The pair-fed animals received amounts of contol diet equal to the quantities consumed by their matched experimental rats. Weight controls were fed lesser amounts of control diet to keep their body weight within 5 g of that of matched experimentals.

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GROUP 3: BIOBEHAVIORAL AND CELLULAR STUDIES ON ETHANOL

Alcoholism: A Speculative View From the Bridge*

Eugene Roberts

Abstract

A global overview of nervous system function under the influence of ethanol is presented. Broad theoretical discussions are provided on the structure and operation of the nervous system and on normal and maladaptive behavior of an organism in its environment.

Introduction

The fact that alcoholism is one of the leading public health problems among civilized nations everywhere suggests that it is a final common path that the susceptible human organism can take when alcohol is available in the environment. An understanding of the nature of this final common path and the many separate roads that may lead to it will require, minimally, an integrated understanding of the wiring diagrams (neuroanatomy and ultrastructure), the electrical and chemical characteristics of the conducting units and transmitting mechanisms in the circuits (neurophysiology and neurochemistry), the interactions between the fast neural systems and the relatively slow endocrine mechanisms (neuroendocrinology), the behavior of the organisms whose activities are regulated by them (psychology), and a thorough understanding of the mechanisms of the actions of drugs (pharmacology).

NOTE: Figures appear at end of paper.

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Keystones of a useful research strategy and a realistic interpretation of experimental results are the recognition of the level at which one is working and the knowledge of the overall structure of the framework at that level (Roberts 1976, 1980; Roberts and Matthysse 1970). In the case of alcoholism, this knowledge is particularly desirable, since what initially may begin with fluidization of membranes of endothelial cells and actions at synapses and gap junctions sometimes may end as a horrendous personal and social problem. In several countries, alcoholism is one of the most pressing public health problems, and inordinate sums are being spent to "combat the problem." What is the nature of the "problem," and how can we "combat" it?

The discussion that follows will indicate the complexity of nervous system function when viewed at several levels and will attempt to imagine the nature of the perturbations that the administration of a drug may produce, particularly one with such ubiquitous effects as alcohol. Ethanol passes through all membranes as readily as water, probably entering all of the cells that it contacts. When consumed, its effects begin in the mucosal linings of the mouth and all regions of the gastrointestinal tract it contacts. Ethanol then continues on to the cellular elements of the blood, the endothelial cells of the cerebral and extracerebral blood vessels, and the ependymal cells, rapidly entering parenchymal cells of all tissues.

Ethanol is a hydrogen-bond breaker par excellence, by virtue of which property it causes the fluidization of membranes. It can exert concentration-dependent effects on the structures of many extracellular and intracellular proteins, influencing their interactions with lipids, polysaccharides, and other proteins as well as with a wide variety of smaller molecular species, including water. The plasma membranes of cells, as well as the membranes of nuclei, mitochondria, and lysosomes, could be affected in a variety of ways, and the rates of movement of substances through them as well as of associated enzymatic processes could be influenced greatly. Associations of structural components of polysomes and Golgi apparatus could be affected, with resultant changes in rates of protein synthesis and intracellular movement, e.g., axoplasmic transport and extracellular secretion of substances such as hormones. Alterations may occur in interactions between adjacent cells and in specific cell membrane recognition sites with neurotransmitters, neuromodulators, hormones, bacteria, viruses, lymphocytes, etc., and in the efficacy of the configurational changes produced by

ligand-recognition site interactions in transmitting signals to associated ion channels or second messenger-synthesizing enzyme complexes. Finally, acetaldehyde, a metabolic product formed from ethanol, may be involved in the further synthesis of compounds such as tetrahydroisoquinolines and β-carbolines, which may exert pharmacological actions of their own (Basmadjian et al. 1978; Blum et al. 1978; Cohen 1978; Cohen and Collins 1970; Davis and Walsh 1970; Hamilton et al. 1978; Myers 1978; Myers and Oblinger 1977). The latter, of great interest in itself, will not be dealt with in this paper.

In the central nervous system (CNS), the site of primary interest to us, the effects of alcohol on membranes of capillary endothelial cells may influence the rate of entry of substances into the CNS by diffusion, pinocytosis, or carrier-mediated transport; or the rate of pumping of K^+ ions out of the brain extracellular compartment may be changed. Direct effects on neural membranes may produce decreases in their conductile properties, changes in release characteristics from terminals of neurotransmitters and modulators, alterations in the sensitivity of pre- and postsynaptic receptors to the action of the latter, and changes in degrees of electrotonic communication between neurons via gap junctions. Brief exposures to single doses of alcohol causing rapidly rising and falling levels in extracellular tissue fluids and in cells would be expected to have effects different from those exerted by prolonged presence of steadily maintained concentrations. In the latter instance, a host of cybernetic adjustments would be expected to take place, structural and enzymatic, so that metabolic steady states different from those found before exposure to alcohol would exist at cellular and tissue levels, and new transactional states would be found at the systems level in the CNS.

In view of the great complexity of the biological situation touched upon above and the ripple-like, radiating effects of alcohol, one would not be surprised to find changes in a myriad of measured parameters in an organism subjected to alcohol. But our task is to attempt to identify events likely to be rate-limiting at each level to be discussed in succeeding sections of this paper and to develop a scenario of events that seem reasonable in terms of available data and that points to some testable hypotheses and, it is hoped, to new ways of dealing with the problem of alcoholism.

The changes produced by alcohol in the behavior of an organism alter its relationship to its physical and social environment, in the long run most often increasing the social stresses to which it is

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subjected. This leads to greater burdens being placed on the neuroendocrine machinery and on the immunologic defense mechanisms, which, weakened by the effects of alcohol, tend to be pushed into overextending their capacities and eventually into failure in the performance of their functions. Continued use of large amounts of alcohol then results in frank pathology in brain, liver, and other tissues.

The simplest and most obvious measure to be taken would be to eliminate the use of alcohol altogether. However, for many reasons, this is an impossibility in the modern civilized world, and furthermore, other toxic drugs and activities would be sought as substitutes. Such a degree of social control would have many undesirable features, and when attempted in the past, has failed miserably. A possibility that has occurred to me is the development of an agent or agents, nontoxic and palatable, that could be required to be added to all commercially available alcoholic beverages that would allow them to retain their sought-after effects while eliminating those that lead to tolerance and dependence. Although this suggestion has met with surprise and incredulity on the part of some authorities in the field, it seems to be a possible goal, given today's biological technologies. Perhaps the first step to be taken is the development of the firm resolve that alcoholism is a problem to be solved and not a way for scientists, physicians, and social workers to make a living.

A General Description of the Organism in its Environment

My working model is a "homing" model, the primary assumption being that the behavior of a healthy waking organism is aimed at attaining a state in which internal homeostatic adjustment is achieved (equilibrium or hedonic state; see figure 1). In the waking organism, this latter condition may be described as the optimal state of well-being and can be associated with comfort, pleasure, ease, satisfaction, absence of anxiety, etc. Displacement from such a state may result in human beings (and possibly in other organisms) in feelings of anxiety, discomfort, boredom, or pain. Continual changes taking place in the external environment and in the internal metabolism of the organism generally act counter to the maintenance of achievement of the equilibrium or hedonic state

and tend to displace the organism away from it. At all times, an awake organism finds itself in a multisensory environment that it scans internally and externally for physical and chemical changes with specialized receptors, and it responds to the patterning of the relative values of the effective sensory cues, an abstraction of the environmental realities. At any particular time, the changing *pattern* in the perceived environment, external and internal, is the stimulus for the organism.

It is presumed that animal organisms possess internal monitoring systems, *the set points of which may vary from one time to another*, that give appropriate signals when the organism is being displaced from equilibrium. Set points may vary with diurnal and menstrual cycles, degree of environmental stress, presence of a disease process, intake of drugs, etc. It is postulated that the level of activity in the monitoring systems is a function of both the external environment (intensity and complexity of stimulation) and the internal environment (activity of drive centers and interoceptive afferents). Perturbations in the monitoring systems, if great enough, may cause an organism to select responses from an array of response options available to it at a given time that will tend to counteract the displacing tendencies. In successful species, the process of seeking this adjustment by individuals of the species gives rises to behaviors that further the survival and reproduction of the individuals and of the species. At any time, the degree of stress experienced by the organism may be presumed to be a function of the extent and rate of displacement from the equilibrium state by all influences acting on it. Both the set points and the degree of resistance to displacement from the equilibrium state may, at least to some extent, be genetically determined. In this respect, it is interesting that recent studies suggest that panic disorder (anxiety neurosis) is a familial disease that is transmitted as an autosomal dominant trait (Pauls et al. 1980).

It is possible that a behavioral option may be chosen that "overshoots" the mark, so that the interaction between organism and environment will cause a bypass of the equilibrium state. An example of overshoot would be the behavior of a neurotic who, anxious as a result of complex emotional stimuli at a party, responds by returning home and going to sleep, or on the other hand, that of a delinquent who responds to boredom by stealing cars. Another example of a response to boredom is that of individuals who, deprived of physical challenges to survival and reproduction, espouse radical causes of the right or left and thus

propel themselves into the violent maelstrom of social conflict in which real threats to their survival may arise (riots, revolutions, etc.). If the stimulus input is too great or complex for the organism to handle at a particular time, maladaptive responses might take place that eventually could lead to the actual destruction of the organism. If the organism survives, a return to an adaptive behavioral pattern may occur through new learning and/or simplification of the environmental input. In the case of humans, psychotherapeutic approaches may be effective, particularly those that disclose the existence and encourage the use of previously unused, potentially adaptive, behavioral options. Also, intervention is possible with drugs, hormones, surgical techniques, electroshock, etc.

Among the potentially restorative options available to humans, and most pertinent to the discussion here, is ingestion of alcohol and drugs. Many of our commonly self-administered, naturally occurring stimulants and sedatives such as coffee, tea, cocoa, alcohol, tobacco, marijuana, absinthe, betel nut, snuff, and spices, and synthetically produced substances such as amphetamines, barbiturates, and benzodiazepines are consumed in attempts to restore the state of well-being. The stimulants are used to overcome the discomforts of "understimulation," and sedatives those of "overstimulation" (see figure 1).

So long as adaptive behavior continues, a search will go on until restoring the feelings associated with the equilibrium state are found. The more restrictive the real or perceived physical and social circumstances, the more likely it is that drug-oriented approaches will be employed. In a dull office environment, it is common to see many individuals with coffee cups and cigarettes in hand at all times. The harassed executive, the busy physician, or the nagged husband or neglected wife may take to alcohol or diazepam (Valium) to give them surcease. Of course, there is much overlap in the choice of agent, and some individuals have learned to titrate themselves with appropriate schedules of "uppers" and "downers."

Every age is the age of anxiety, if anxiety is defined as the perception of being displaced from an equilibrium or hedonic state (figure 1). For eons, humans have sought external aids in alleviating the pangs of suffering inherent in this perception. Alcohol has been the most common and widely employed antianxiety agent. A recent quote from a U.S. Congressman (Los Angeles *Times*, March 2, 1981, p. 1) is frightening to the citizen and illustrates one of the

uses of alcohol. "You're just a piece of meat in this business. People are always pulling at you to do something for them. A man needs to get off this treadmill—and alcohol is often the easiest way to escape."

Once behavioral options are discovered that, by themselves or by facilitating the use of preexisting ones, are effective in helping an organism to successfully counteract the effects of equilibrium-displacing factors, they are highly reinforced and rapidly incorporated into the behavioral repertoire. Since the internal experiences of the organism and the chemical and plastic changes in the CNS that accompany them probably are similar, whether they are purely behavioral or drug associated, it is not surprising that the phenomena of tolerance, dependence, withdrawal, and ready recidivism after brief abstinence can be associated with both. The latter hallmarks of alcohol and drug addiction also may be applicable to such phenomena as gambling, masturbation, overeating, gang membership, television viewing, and dependence on religious ritual and on particular close interpersonal relationships. It is, therefore, no surprise to me that the "cure" for drug or alcohol addiction often is accompanied by the adoption of rigid and ritualistic religious beliefs or adherence to other types of highly structured oxthodoxies that may be imposed on any aspect of living, e.g., political, interpersonal, dietary, etc. Indeed, to paraphrase an old Marxist dictum, it can be said that "orthodoxies, including Marxism, can become the opiates of people"—a statement that makes a good deal of sense in neurobiological terms. I believe that we will truly understand alcoholism when we find the common thread linking the above. I have heard about the experiences of a man who, in his youth in the mid-1930s, left Utah as a devout Mormon following completion of 4 years of college and his 2-year mission and went to Columbia University in New York City to obtain an advanced degree. As his faith in his religion began to crumble under the onslaught of liberal thought to which he was being exposed, he began to experience sweats, shakes, and nightmares and frequently felt that he was about to die. If the description of his symptoms were related without giving the context, one might easily conjecture that he had been a drug addict and was being withdrawn "cold turkey."

The Through-Put and Cybernene Neural Systems

When an organism perceives that a problem exists, the stimuli reflecting the problem simultaneously release both through-put (dark lines) and auxiliary neural circuits (light lines) (figure 2). The through-put circuits are envisioned to relate to the auxiliary circuits much in the way that camshafts in some automobile engines are related to the oiling system. The oil pump is driven by a gear connected to the camshaft. The faster the camshaft turns, the more oil is pumped to the movable parts of the engine. The through-put neural circuitry may be considered to consist of cascades of serially alined neuronal assemblies in which coded patterns of information entering originally from sensory transducers are progressively refined by the reduction of redundancy and the selection of particular features. The transformations of coded patterns in different neural sectors are achieved to a considerable extent by negative feedback loops that exist between and within the sectors. The "hard-wired" neuronal elements of the mainline through-put nueronal circuits, the blueprints for which are largely inherited by the organism, are surrounded by local circuit neurons whose specific commitments may be made during development as well as later in life, and which not only participate in virtually all phases of information processing but also may undergo the plastic changes that must be involved in long-term retention of experience. Communication between the neural elements in these circuits takes place through synaptic and gap junctions on a millisecond or submillisecond time scale.

Elements of the auxiliary system release substances (cybernenes) that are the "oil" required for the smooth functioning of the neuronal machinery. That is, the effect of the neurons' releasing such substances may be analogous to that of squirting oil into inadequately lubricated but intact machinery, the parts of which will not function properly if either insufficient or excess oil is furnished. However, the oil is not part of the actual machinery. I would like to suggest that in many instances in which they act in the CNS, acetylcholine, catecholamines, serotonin, neurally released peptides, and prostaglandins may serve to optimize regional nervous system activity in relation to functional demands without themselves necessarily being involved in specific information transmittal. Upon release from nerve terminals, they exert chemi-

cal actions that influence the efficacy of the information-transmitting junctions in the mainline circuits. Such substances, which influence neural function without themselves necessarily being involved in rapid neurotransmission, are called cybernenes, according to the suggestion of Roger Guillemin. For example, immunocytochemical, isotope labeling, and physiologic experiments are compatible with the suggestion that norepinephrine neurons in rat brain largely perform global, hormone-like functions, although occasionally they also may participate in typical synaptic relationships. Recent experiments have shown that norepinephrine, but not dopamine, can enhance neuronal responsiveness to both GABA and acetylcholine on neurons of the rat somatosensory cortex (Waterhouse et al. 1980). It is interesting that acetylcholine is a "true" transmitter at nicotinic vertebrate neuromuscular junctions but appears to exert modulatory actions on both inhibitory and excitatory synapses of a muscarinic agent in the vertebrate CNS. In general, the cybernenes may exert relatively long-lasting effects on the cellular elements, possibly setting the gain on the efficacy of individual synapses, on specific types of synapses, or on all of the synapses in a given region. Their facilitatory effects, often exerted via the cyclic nucleotides, may be exerted at many functional loci, such as transmitter synthesis, the postsynaptic control of ion channel open time after transmitter impingement, and the setting of windows on the range of potential firing frequencies that may be employed by given neurons or groups of neurons. These effects could be exerted by a large variety of cascading molecular mechanisms.

An inappropriate balance between availability and distribution of such cybernenes and activity in through-put neural circuits could result in gross malfunction of the CNS, as is found in Parkinson's and Huntington's diseases or in schizophrenia. It is striking that when the substantia nigra is stimulated, physiologically recorded signals in the corpus striatum do not seem to be greatly altered when the nigrostriatal dopamine neurons are destroyed by 6-OH-dopamine or when the action of dopamine is blocked completely by large doses of haloperidol. Also, the spontaneous firing rates of cells in the caudate nucleus are not altered by dopamine-depleting lesions of the nigrostriatal pathway. This suggests that the physiologically relevant signals largely are carried by fibers of nondopaminergic nigral neurons and that the effects of dopamine released from the dopaminergic fibers are not informational in the strictest sense of the word. A phenomenon related to the above is

described in many anecdotal accounts about parkinsonian patients, obviously suffering from a defective functioning of nigrostriatal dopaminergic neurons, who can fully mobilize normal and adaptive physical activity in an emergency but who relapse into the typically inactive parkinsonian state as soon as the emergency is over. The above and the therapeutic effects of exogenously supplied levodopa in parkinsonism are compatible with the suggestion that the neuronal "hard-wired" circuitry in the neostriatum is potentially available and that the dopaminergic neurons furnish dopamine, the "oil" required for the neuronal machinery to function smoothly. In contrast, it seems likely that relative overactivity of the dopamine and/or the noradrenergic systems in particular brain regions is associated with psychotic and schizophrenic disorders, and the favorable actions of neuroleptic drugs and propranolol (Roberts and Amacher 1978) in some instances may be attributable, at least in part, to their dampening of the effects of these systems by receptor blockade.

From the preceding discussion, it can be seen that the cybernene and through-put systems can be considered as entities having somewhat different but complementary functions in the CNS. They are mutually interactive in all instances and in all neural regions. The main idea is that when an organism first perceives that a problem exists, the stimuli reflecting the problem release both through-put and cybernene circuits simultaneously, the latter helping maintain a general state of readiness at the outset. In other words, at this early stage, some of the cybernenes may act as general sensory and response amplifiers. In addition to the neurally released cybernenes, a variety of pituitary and adrenal hormones may be released into the bloodstream on signals originating in the CNS, in this way altering membrane properties and metabolic states of various tissues, including those of the nervous system. The endocrine and neural events also are mutually interactive at all times. Probably the cores of the cybernene systems are located largely in brainstem regions. For example, cholinergic pathways from magnocellular nuclei of the basal forebrain and noradrenergic pathways from the locus coeruleus fan out similarly to terminate in various cortical and subcortical loci, often after long traverses away from their cell bodies.

The points at which structural or functional lesions or both can take place that would impair the cyberneticity of such a system are legion. It is, therefore, no surprise that every known transmitter candidate and every potential cybernene under one circumstance

or another has been thought to be involved in various types of neural dysfunction. The literature on alcoholism has dealt with all such known agents, putting emphasis on one or another, as the fashions wax and wane.

A Guided Tour Through a Model of Behavior

Experience of the external and internal environment can occur only through the sensory receptors, which are acted upon by physical and chemical changes. The proprioceptors sense what goes on in the muscles, tendons, joints, mesenteries, and blood vessel walls of the organism itself. The exteroceptors and the interoceptors deal with occurrences outside the organism, the former monitoring the outside world from the surfaces of the organism and the latter giving information about events largely concerned with the processing of food in the mouth and in the digestive cavity. Much work has been done and is in progress with regard to the mechanisms by which receptors transduce environmental changes into coded signals usable in the nervous system and the ways in which receptor sensitivities are controlled. The sensory information undergoes neural transformation (partially innate and partially learned) at various levels in the nervous system in such a way that the organism experiences an abstraction of reality, not reality itself. Animal organisms often react to acoustic and optic symbolic equivalents of environmental stimuli, a subject extensively explored by ethologists. Humans make use of verbal symbols; thus, the threat of loss of a job may be equated with a threat to life, the possession of money and material things to food or sex. The characteristics of the neural circuits in some of the pertinent systems have been described, and the types of information processing and the transformations that take place at various levels from the receptor to the cortex are being studied. The cellular units of the nervous system, the neurons, glia, and endothelial cells, are being ever more thoroughly described from morphological, physiological, and biochemical points of view, and the modes of communication between neurons, synaptic and electrotonic, are being elucidated. Neurons transmit both excitatory and inhibitory information, and the manner in which inhibition and excitation interact in the function of a particular neuronal system now can be illustrated in considerable detail by our knowledge of how informa-

tion is processed in such diverse structures as the cerebellum of the vertebrate brain and the stomatogastric ganglion of the spiny lobster.

The nervous system can abstract, from the unanalyzed stimulation impinging on the receptors, qualities of the perceived objects that are more relevant for behavior than the unprocessed signal itself. Thus, in the visual system, similarity and contrast, onset and termination of stimuli, and motion and geometrical shape are singled out for perceptual attention. However, a still higher level of perceptual integration must take place before the organism can respond adaptively to the pattern of sensory input. The stimulus pattern must be brought into relation with memories of past stimuli and their consequences (associative integration) and with the drive state of the organism (emotional integration). In other words, some information processing system must call up from memory storage stimulus patterns similar to the one at hand and decide whether or not the stimulus presents opportunities for need satisfaction or potential threat to survival.

Although the mechanisms of this higher order perceptual integration are unknown, evidence suggests that some of the integration takes place in the hippocampus. For example, hippocampal ablation in monkeys causes failure to recognize previously familiar objects, and hippocampal seizures cause amnesia. In humans, the loss of recent memory in Korsakoff's psychosis, which involves lesions of the circuits associated with the hippocampus, may be caused by the inability to form associations between newly presented stimuli and old memories. In memory loss after hippocampal lesions, amnesia may be most severe for memories that have a strong emotional component. The hippocampus is invariably affected in senile dementias and Alzheimer's disease, regardless of the other regions of the brain that may be affected, and is known to be damaged in chronic alcoholism. Prolonged ethanol consumption has been shown to produce profound morphological changes in the hippocampus—losses of dendritic spines on hippocampal pyramidal cells and dentate granule cells and a significant loss of hippocampal pyramidal and dentate gyrus granule cells (Riley and Walker 1978; Walker et al. 1980). It also is likely that the functions and structures of GABAergic interneurons (basket cells and horizontal interneurons) in the hippocampus, aspinous stellate cells in the cortex, and GABAergic neurons in other neural structures also are seriously disturbed with prolonged alcohol intake (McMullen et al. 1980; Nestoros 1980; Nestoros et al. 1980; Pittman and Siggins

1980; Rogers et al. 1980; Ticku and Burch 1980). There is sufficient experimental evidence to suggest that alcohol consumption, even for a relatively short period of time in the life of an organism, can create lasting disturbances in neural structure and function, so that the reserve capacity to withstand subsequent insult, whatever its nature, might be reduced.

Since alcohol is the most common toxic substance known to lead to dementias when taken in large quantities over a long period of time, it is highly desirable to determine whether or not use of alcohol is in any way correlated with the extent of occurrence of Alzheimer types of senile dementias or with their time of onset. Indeed, chemical measurements suggest that there is some commonality in the changes that occur in the brains of individuals suffering from senile dementia and those of chronic alcoholics (Carlsson et al. 1980). Are senile dementias of the Alzheimer type more frequent, or do they occur earlier in countries with relatively high intakes of alcoholic beverages, such as France, Italy, and Ireland, than in Japan and Israel, where per capita alcohol intake is said to be comparatively low? What are the statistics for the Mormon population of Utah, supposedly a largely nonimbibing group, compared with the non-Mormon population in the same State?

An abundant stream of fibers emanates from the hippocampus to the hypothalamus by way of the fornix, making it possible for the integrated stimulus pattern, invested with its full associative and emotional significance, to be brought immediately into interplay with the neuroendocrine system. Defective hippocampal function would thus give rise to maladaptive neuroendocrine responses. Virtually every endocrine function studied has been found to be disrupted by alcohol, at least to some extent. The hypothalamo-hypophyseal system is particularly responsive to alcohol. The release of vasopressin and oxytocin is inhibited by alcohol (Gross 1975; Majchrowicz 1973). In this connection, it is of great interest that arginine vasopressin, a hormone synthesized entirely within the hypothalamus, and some of its structural analogs can augment memory functions in animals and humans (see Beny and Baertschi 1981; Weingartner et al. 1981; and references cited therein). Adrenal atrophy and abnormalities in rates and extent of release of adrenal cortical hormones have been reported to result from alcohol administration (Gross 1975; Majchrowicz 1973). Some permanently deleterious effects of temporary but heavy use of

alcohol may be expected on endocrine function and neuroendocrine relations.

The neuroendocrine servo system (NES), or the hypothalamo-hypophyseal system, is a part of the central visceromotor system, which in turn may have important influences on behavior, particularly at times when a new environmental setting requires the organism to develop new ways of adjustment. The key to the NES appears to be in the hypothalamus, which plays a central role in the coordination of physiological processes within the organism so that a relative constancy of the steady states (homeostasis) within the organism is maintained at all times. The imperfection of the blood-brain barrier in the hypothalamus makes nerve cells in this region much more sensitive to humoral influences, and probably to alcohol, than are those in most other regions of the CNS. Changes in levels of most blood constituents appear to have an effect on some specialized neurons in the hypothalamus.

The NES and the limbic nervous system that controls the NES receive inputs representing the emotionally significant aspects of the total stimulus pattern, external and internal, and summate them. The neural representation of these signals may be the frequency of discharge of neurons in certain neural regions. Or there may be a more complicated index, such as the degree of correlation between discharge times of groups of neurons taken at random in given nuclei—in other words, an index of the degree of synchrony. Two kinds of restorative processes may be initiated when the deviation and/or the rates of change of deviation from a preprogramed optimal level, perhaps the physiological equivalent of motivation, exceed a certain value. Initially, a signal is generated that causes the organism to act according to the response options already available. These options are stored as fully programed, hierarchically arranged, integrated sequences of behavior in various parts of the nervous system, ready to go into effect when released but held in check by inhibitory command neurons (Fentress 1976). These behavioral sequences are normally in a state of inhibition, except perhaps for occasional spontaneous release that gives rise to a low background of unstimulated display of behavior. The releasing signals act mainly by inhibiting these inhibitory command neurons, resulting in the release of the pacesetter neurons of whatever behavior patterns are "prepotent," that is, those at the top of the hierarchy of options at that time. In addition to this behavior-releasing function, neurohumoral changes occur that may facilitate plastic changes locally or throughout the

CNS. These effects may include changes in local circulation and capillary permeability as well as the secretion of hormones and growth factors, putting the organism in a condition of optimal readiness to learn new adaptive behaviors. The result of the impingement and processing of information is a behavioral response—the reaction of an organism to the environment or action upon the environment, or both. Detailed analyses are being made of the effector systems, the neuronal systems that give signals to muscles, glands, etc., or modulate preestablished spinal reflexes.

Learning and Habituation

An organism reacts to a familiar environment with responses learned in previous encounters with the same or similar situations. For convenience, we can think of two broad categories of learning: (a) an organism learns to do something to modify the environment, and (b) it learns to cease to respond to the environment (habituation). In the case of an actual organism adjusting to its real environment, both kinds of learning usually are involved, the relative amounts varying from situation to situation. For purposes of the following discussion, the two types of learning will be treated separately.

When an organism is presented with a new effective stimulus setting, which consists of a perceived pattern of physical and chemical changes in an environment (external plus internal), receptors are activated in a unique fashion (figure 2), i.e., the types and numbers of receptors activated and their sequence and intensity of activation result in a receptor and neural activation pattern different from any experienced previously by the organism. Even in a well-controlled experiment in which only one sensory modality is manipulated by an experimenter, it is the changing pattern of the environment that is the stimulus for the organism. The stimulus value of a red light, for example, depends on the context in which it is embedded (e.g., a traffic intersection or a house of ill repute). As a result, the monitoring system that I presume to exist is put into action, and the normal healthy organism reacts through its effectors so as to restore the equilibrium state (figure 1). A good monitoring system for a multicellular organism might consist of some strategically located spontaneously firing cells or groups of cells (oscillators), with excitatory and

inhibitory multisensory inputs, whose firing patterns at the equilibrium state would be poised at a level that would give maximal detectable change with minimal change in input. I would like to consider the possibility that the approach to the equilibrium state could be signaled by the synchronic firing of strategic groups of neurons in one or more oscillators, with phase-locking occurring between oscillators, and that displacement from equilibrium would be indicated by desynchronization of the firing within and between these potential oscillators (see Glass and Mackey 1979*a*,*b*; Mackey and Glass 1977). There could be a delicate balance between synchrony and asynchrony in these oscillator loci, conferring the requisite sensitivity to the detection devices. This concept will be discussed further in other sections of this paper.

The subsequent course of the behavioral options employed is determined at least in part by whether the behavior can lead to the modification of the environment or adjustment to it, in such a way as to increase the sense of well-being of the organism, as reflected in the approach of the firing patterns of the cells in the monitoring system to that characteristic of the equilibrium or homing state. If action on the environment is possible, stimulus intensity or complexity might be decreased by eliminating the life-threatening or noxious situation or obtaining food, water, sexual satisfaction, etc., or it might be increased by various types of exploratory activity. Each recurrence of a particular stimulus set gives opportunity to the organism to achieve behavior that is more effective in terms of acting appropriately in response to a given environmental setting and that is more efficient and economical in terms of the resources of the organism.

These learning processes are accompanied by increases in connectivities in the CNS of those neural pathways that set into action the appropriate combinations of effectors (behavior). Increases in connectivity of inhibitory connections may be at least as important as facilitation of excitatory ones. The word "connectivity" as applied to neural pathways is used to refer to the probability that a particular pathway will participate in a response to a given environmental setting. Neural circuits can inhibit the receptor signaling systems that activate them, and effectors can inhibit the circuits that set them into action. It may be that these inhibitory connections are differentially strengthened by learning, so that the organism learns to attend to the most relevant sensory cues and to inhibit interfering responses; learning to play a particular note on the piano with one finger may involve learning not to play the

other 87 notes with the other nine fingers. At least some of the effector organs can liberate substances into the blood or remove substances from it, so that changes in concentrations of these substances may constitute inhibitory feedback signals to the hormone-secreting cells activated by the stimulus. For example, release of cholecystokinin or bombesin from the intestine and/or in the brain during food intake may give satiety signals by combining with receptor sites in the hypothalamus and olfactory bulb.

Habituation may take place when the organism cannot act on the environment to change or modify the stimulus. From the point of view of the organism, this would be the most effective way of dealing with a situation that cannot be altered. There would be no possibility of achieving a new coordinated behavioral (or effector) pattern appropriate to the continuing stimulus; and the behavior observed initially, and for as long as overt responses to the stimulus could be observed, would generally be of the "alarm" type indigenous to the species studied. For a period after the first experience with the stimulus, more primary sensory neural circuits would be active than would be the case if the stimulus intensity were reduced by effective behavior. As a result, more neural circuits could undergo plastic changes, developing strong negative feedback connectivities with the receptor signaling systems, and in the limiting case a state could be achieved in which the particular stimulus pattern to which the organism is exposed after the first fraction of a second would evoke some activity in the CNS, but there would be no overt behavioral response and little or no activation of the hypothalamo-hypophyseal endocrine responses.

We are all familiar with situations in which we become accustomed or habituated to sights, smells, or sounds or to the mannerisms of other people that are at first annoying. There are some experimental data that can be considered with regard to the physiological mechanisms possibly involved in habituation. Experiments were performed with cats (John and Killam 1959) in which there were chronically implanted electrodes in the following areas: visual and auditory cortex, mesencephalic reticular formation, superior colliculus, amygdala, posterior hippocampus, lateral geniculate, and anterior hippocampus. When the animals were first exposed to flickering light of a given frequency, responses having the frequency of the light stimulus were observed in all areas in which the electrodes were placed. After a number of days of "familiarization" with the stimulus, the responses decreased markedly in all areas. After 20 days, there was virtually no evidence of

following in the visual cortex, reticular formation, and hippocampus; even in the lateral geniculate body, in which originally there was continuous frequency-specific following of a relatively high amplitude, only occasional small residual bursts of very low amplitude were observed. In general, centrifugal inhibitory control systems may be operative for all sensory modalities.

All of us know from experience that habituation sometimes can be a difficult and prolonged experience. Wide variations exist in the capacities of individuals to habituate to stimuli and situations in which action is not possible. Alcohol often is used by sensitive persons to assuage pain by decreasing the intensity of the sensory input, which alcohol might help achieve by decreasing presynaptic release of neurotransmitters from terminals of primary afferent nerves (Carmichael and Israel 1975; Erickson and Graham 1973; Faber and Klee 1976; Sinclair and Lo 1978; Subramanian et al. 1978).

The net effect of the maximal development of connectivities in ordinary learning and habituation, upon continued experience of an organism with a particular stimulus set, would be the establishment of a cybernetic system, different at least in some respects from any present in the organism before, in which after the first moment of stimulation the activation of the minimal number of neural circuits and the minimal release of hormones would be accompanied by behavior that would approach the maximal efficiency attainable by the organism under the circumstances. Herein would be the essence of a system with a defined structure that has remarkable self-organizing and plastic properties.

A Behavioral Basis for Addiction

I postulate that the synchronous firing of specific phase-locked neuronal oscillators is the signal to the organism that achievement of the hedonic state is in sight and that appropriate behavior options are about to be released that will solve or ameliorate the problem that has given rise to feelings of anxiety and will bring it to an end. The performance of innate behaviors may, in itself, serve to reinforce responses during a learning situation. Examples of such behaviors in all animal species are eating, drinking, and copulating. Burrowing and gnawing in rats, singing in birds, and attacking in cats are examples of species-specific behavior. One can

describe a set of characteristic responses which are adaptive for particular species. These responses can be categorized as those which bring the animal into contact with stimuli relevant to its survival (approach) and those which remove it from stimuli which are threatening to its survival (withdrawal). These species-specific response sequences are part of the organism's evolutionary heritage. If one accepts the biological wisdom of evolution, it is not circular to suggest that the performance of these approach and withdrawal sequences are, respectively, positively and negatively reinforcing.

> A selective review of the physiological, behavioral, and anatomical literature led us to describe a mechanism whereby *reinforcement consists of activation of neural systems located in the brainstem which mediate the expression of these specific responses* [italics added]. Reinforcers, accordingly, are those stimuli which are capable of facilitating the activity of these systems. Not all stimuli would be equally capable of this facilitation, and presumably the conventional reinforcers of behavioral psychology are those which bear particular facilitatory relationships to the different response systems (Glickman and Schiff 1967).

What are the above postulated neuronal groups, where are they located, and what is their modus operandi? A key to the loci of the monitors referred to above may have been furnished by the work begun by Olds on the "reward" centers of the brain and continued by many others (Hall et al. 1977; Olds 1977). There are sites in the hypothalamus and brainstem that, when stimulated electrically, appear to be so rewarding that given the opportunity to continue the stimulation by pushing a lever making the electrical contact, the implanted animal will choose to do this over any other activity. There appear to be several such relatively discrete regions in the brains of rats (and probably all other creatures), not one. Although the neuronal groups have not been well defined, despite much work, I presume them to be part of a communicating group of neural oscillators whose phase-locked activity under ordinary circumstances communicates the signal to the organism that the equilibrium state is attainable, and that appropriately coupled behavioral options are about to be released to achieve the consummation of the state. It is not surprising that these reward centers often have been found to be associated with regions governing activities related to consummatory responses, i.e., food and water intake, attainment of sex, etc.

I now would like to suggest that the postulated neuronal groups, in order to give this signal, must undergo a transition from asynchronous activity, in which the neuronal constituents, the

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neurons, largely are firing at individually determined rates, to a synchronously firing state in which they are firing essentially as a unit. In the latter state I presume the neurons in each oscillator to be communicating with each other electrotonically as well as synaptically, whereas in the former state they largely are communicating through chemically conducting synapses. Once one of these groups begins to oscillate, it can cause the others to oscillate in a phase-locked manner. In other words, I postulate there to be a hedonic network of oscillators, the members of which can engage each other in phase-locked oscillatory activity. One consequence of the latter type of activity may be a considerable inhibition of all types of sensory input and/or an insensitivity to sensory signals during its duration. The postulated network is presumed to be an anticipatory one, indicating that the consummatory acts are imminently attainable and that the hedonic state is achievable, perhaps reaching the maximal degrees of synchrony just before or during the consummation, as, for example, in sexual orgasm. This may be the basis for the poetic vision of paradise. However, once achieved, the neural and chemical inhibitory feedbacks cause the reversion of activity within and between the oscillators to a nonoscillatory type, and this might be associated with the despair of paradise lost. In a coupled system, such as ordinarily exists in an animal facing real problems of existence and achieving realistic solutions to them, it is posited that the system operates in the manner described. However, the oscillators can be made to operate independently of the usual environmental signals, such as by electrical stimulation or by drugs. Whether produced by approach to solutions of existential problems or by drugs or artificial brain stimulation, I propose that these oscillations, at least in human beings, are accompanied by a temporary euphorigenic rush of feeling, or a "high." The substances or conditions giving rise to the oscillations become associated with this type of feeling and may become relentlessly sought after. The latter may be considered to be a basic characteristic of most human beings.

As one physicist, famous for his succinct statements, has said, "There are three important things in life to me, food, sex, and ideas; and they come in that order." Those of us who are fortunate can get some "highs" from symbolic equivalents or abstractions of the basic elements of survival and reproduction. Thus, approaches to the solution of difficult problems, whatever their nature, may give the "highs" that commit individuals, literally addict them, to particular activities. It is biologically immaterial to the individual whether

he or she pursues scientific, artistic, or literary goals or seeks relentlessly for wealth, power, and seduction or dominance of others. Unfortunately, the social consequences of the choices made and the nature of the means employed to attain the related goals are unpredictable and sometimes may be disastrous. A frequently asked question is why the achievement of some individuals in terms of accumulating wealth, exercise of power, rewards of fame, etc., are not enough. Why is there no feedback? This may be equivalent to asking why an alcoholic who has drunk an ocean of alcohol or a drug addict who has injected pounds of heroin into his veins does not stop. What is sought so persistently is the temporarily achieved vision of paradise that may reside in the oscillation of certain nerve circuits and not the product of the activity that results from the means of achieving this effect. Paradoxically, neural devices, which were selected by nature as being of key importance for survival of our species under the primitive conditions that existed for several million years, may become the means of destruction of all species in a nuclear Armageddon, as the power addicts in the Kremlin and the Pentagon rationalize their abstractions.

The first addiction may be to the mothering person. At the beginning of its life, a human infant experiences intense feelings of hunger, thirst, and needs for warmth and dryness. The nervous system and the organism in general are far from adequately developed to satisfy any of their own needs. Originally the sequence may be presumed to be something like the following: A sense of displacement from equilibrium (anxiety) is signaled by neurons in the brain receiving specific information about hunger contractions in the stomach and fall in blood sugar as well as by more general alarm signals that may be caused by increased blood levels of pituitary and adrenal hormones. For the infant, this state initially must be close to the ultimate in anxiety, and the appropriate behaviors, e.g., crying, muscle tension, and distressed facial expressions, are released. The mother then enters the room, lifts and fondles the baby, and offers it the breast. The touch of the breast on the lips releases the sucking reflex. As soon as the warm milk begins to enter the mouth and stomach, the firing of the neurons indicating the degree of displacement from equilibrium will decrease and the synchronic activity of those in the hedonic oscillators will begin to take place. As feeding proceeds, the stomach begins to fill, the blood sugar begins to rise, insulin and glucagon are secreted, and chemical signals released peripherally and centrally may signal the end of hunger to appropriate cells in the

hypothalamus and possibly other brain regions. The concatenation of neural and chemical signals arising from these and a myriad of related events become the inhibitory signals to the hedonic oscillators, desynchronizing their activity.

As time goes on and the nervous system develops, learning takes place so that the oscillators are put into action by the various sensory signals emanating from the mother, such as odor, touch, sound of her voice, and even her heart beat. Eventually the sound of her step, the opening of the door, her footsteps in the hall, and even the time since her last appearance may have adequate signal value. Finally, even in the absence of need, and in absence of the mothering person, a picture of her, or even the thought of her, may set the oscillators going. Thus, with the internalization of "mother," the ability to evoke the associated sensory experiences may give individuals the capacity for activating their own hedonic oscillators in a manner not dissimilar to what would occur if, by pushing a lever, they were to activate electrodes placed near one of such oscillators in their brains. Thus, a deep association of mother, in reality and in thought, with the attainment of basic survival needs and the related innate consummatory neural circuitry must take place. Even at an early age, the circuits subserving sexual functions and needs are occasionally disinhibited. Their ease of release increases greatly at puberty. Here again, the close proximity of the mothering person may associate this individual, as such, or some of her abstracted characteristics, with the oscillator or oscillators related to sexual circuitry. This can quite naturally result in her becoming the maximally desired sexual object for a long time to come, which is a genetically and socially maladaptive occurrence. In recognition of this, taboos, laws, and religious customs have been devised to make incestuous liaisons and even thoughts illegal, immoral, and often punishable by severe measures.

As a result of the inordinately long period of dependence of the human infant, such strong attachments to the mothering person may take place that sudden separation (weaning or leaving home) may result in symptoms that closely resemble those seen on withdrawal from alcohol, morphine, or tobacco. In this connection, it is of interest that the drug peddler in the movie "A Hatful of Rain" was called Mother by his customers. In most cases the experiential exposure outside the home or in an extended family gives the growing child opportunities to achieve other attachments that tend to dilute those to the mother and that eventually allow

the substitution of other individuals or internalized concepts in association with the basic survival and sexual circuits. Most religious and orthodoxies have some mothering aspects to them and, when inculcated early, can serve the purpose of weaning away from the real mother; but they may become addictive, themselves, as indicated in a preceding section. There is little question that the early attachment to a mothering figure, interacting with a host of genetic and developmental variables, plays an important and sometimes dominant role in many of life's subsequent choices and in reactions to many of its events.

The above discussion, grossly inadequate and incomplete, serves to indicate that I believe there is a fundamental connection between addictability of human beings to a wide variety of substances and circumstances and the basic characteristics of the structures of the nervous system of an organism and its developmental history. It seems as though the nervous systems of all mammals, at least to some extent, share the characteristics that make it possible to become addicted to morphine and alcohol, since, under suitably chosen conditions, even mice have been shown to exhibit the characteristic combinations of tolerance and dependence.

What Are Neuronal Circuits Like in Principle?

The Communicating Units

The functional units of the nervous system are the cells that make up its structures (neurons and glia), the blood vessels that supply the regions in which these cells are found, and the chemically transmitting synapses or gap junctions through which the neurons communicate. Neurons typically have four major identifiable regions: dendrites, cell body (soma or perikaryon), axon, and specialized axonal endings. Much of the communication that takes place between receptor and neuron, between neuron and neuron, and between neuron and effector cell is believed to occur via the extracellular liberation of a substance or combinations of substances that interact with specialized regions of membranes of neurons or membranes of muscle or gland cells to produce either excitatory or inhibitory effects. The key to the action of transmitter substances lies in the nature of the changes they cause in the

conformation of receptive membrane regions on neurons. A transmitter is neither excitatory nor inhibitory in itself, but only in relation to a particular membrane with which it interacts. Thus, a given neuron may liberate a particular transmitter from its axonal terminals onto the membranes of many other neurons. In some instances, the transmitter may exert excitatory effects and in others, inhibitory effects. The demonstration that at a given synapse GABA or acetylcholine is released, for example, is not sufficient evidence to identify it as an inhibitory or excitatory synapse, respectively. Supporting physiological evidence always is necessary for such a functional assignment to be made. Acetylcholine is always excitatory when it interacts with nicotinic receptors at neuromuscular junctions, and it is inhibitory when liberated from brainstem neurons onto muscarinic receptors of cells in the reticular nucleus of the thalamus.

Although not yet thoroughly analyzed at the physiologic and ultrastructural levels in most regions of the vertebrate nervous system, electrotonic interactions through gap junctions also may be of key importance in complex information processing (Bennett and Goodenough 1978). The formation and dissolution of electronic junctions between neuronal processes may be occurring continuously, and alterations of environmental factors, e.g., extracellular levels of calcium ions, pH, and membrane potential, may play a role in determining the extent and stability of such junctions and the numbers and types of neurons that are linked through such junctions at any given time. Their activation or deactivation may play a key role when transitions take place from asynchronous to synchronous activities in groups of neurons. Such junctions may also play important roles in the transfer of molecular information from one neuron to another, allowing neurons to inform each other about their metabolic states and possibly to coordinate their metabolic activities.

In the case of a given neuron, substances that are liberated upon its dendrites, soma, and initial axon segment from specialized endings of other neurons exert excitatory and inhibitory effects upon it. Excitatory effects upon a neuron occur most frequently on dendrites. The action of an excitatory transmitter is believed to result in a configurational change in the membrane upon which it impinges, which increases its permeability to cations and in turn decreases the potential across the membrane (depolarization). When a neural membrane is depolarized by the impingement of an excitatory transmitter or by some other means, the relationship of

calcium ions to membrane components is altered in such a way that, in addition to the inward flow of calcium, there also may be a closely related inward sodium flux. The ionic currents thus established propagate actively toward the cell body, depolarizing adjacent regions as they advance. Although the sodium current usually is responsible for most of the observed depolarization of the postsynaptic membrane, the increase of free intracellular calcium that may occur from inward flow or from release from mitochondria during nerve activity activates the opening of potassium channels. The outward potassium current then serves to repolarize the cell and, in many instances, to produce a hyperpolarization before the calcium balance is restored via the action of Ca^{++}-MgATPase and mitochondrial reuptake, and the potassium channels are closed. The action of Na^{+}-K^{+}ATPase restores the monocation balance. Sometimes the latter pump may overshoot the mark and result in hyperpolarization, a condition resulting in neural inhibition.

The increase in free intracellular calcium, brief as it may be, also is believed to trigger the sequence of events that is important for both the release of neurotransmitters from nerve terminals and the release of the metabolic reactions required for recovery from nerve activity and for the possible retention at pre- and postsynaptic sites of a biochemical "memory" of the experience. During this period, various enzymes related to cyclic nucleotide metabolism are activated as well as phosphoprotein phosphatases and phosphokinases. As a result, there are alterations in degrees of phosphorylation of membrane components and enzymes, with resulting release of metabolic recovery reactions, alteration of membrane affinities for anions and cations, and, in general, occurrence of cascades of interdependent reactions throughout the cellular machinery. These reactions may produce short-term or long-term changes in the activities of neurons.

Inhibitory transmitters, which most often are liberated on dendrites close to the cell body, or on the cell body itself, increase the permeability of the membrane to anions (particularly chloride) and cause it to resist depolarization. Inhibitory mechanisms accelerate the rate of return of the resting potential of all depolarized membrane segments that the transmitter contacts and stabilize (decrease sensitivity to stimulation) undepolarized membrane segments. GABA, the major inhibitory neurotransmitter in vertebrate organisms, typically produces an increase in membrane permeability to chloride ions that is measured as an increase in

all. Glial cells may serve as one major restraining influence on spontaneous activity of neurons. This would be done by removing substances from the extraneuronal environment in the regions of synapses, by adding substances to it, by preventing diffusion of substances to it, or by preventing diffusion of substances liberated from neurons in such a way as to shunt depolarizing ionic currents, thereby decreasing intrinsic excitatory levels of neuronal membranes below their spontaneous firing levels. Another type of inhibitory influence is exerted by the effects of neurotransmitters liberated onto neurons from inhibitory neurons. Such inhibitory neurons could be only phasically active, with their activity depending on the inputs to them; or they could be tonically active, spontaneously firing cells. Some inhibitory neurons might release inhibitory transmitter constantly without an action potential, the rate of release being determined by the degree of membrane polarization. Release from inhibition could be achieved by direct depolarization of the inhibited cell, by inhibition of the inhibitory neurons, or most commonly, by a combination of both.

In the past, neuronal circuits were considered almost entirely in terms of excitatory events. The first neurons in the circuits were presumed to be excited by some input and to pass on excitatory or depolarizing messages synaptically in such a way that there would result a progressive excitation passed from neuron to neuron, until the final neuron in the circuit would depolarize an effector cell, muscle, or gland. Inhibition was considered to play, at most, a vague modulatory role. Only within the past 30 years have the major and essential roles of neural inhibition and disinhibition in nervous system function become fully recognized.

Disinhibition—A Major Organizing Principle

Inhibitory and excitatory neurons participate together in information processing in such a way that it is possible for particular organisms to respond adaptively to their environments in a manner compatible with survival and successful reproduction. The ubiquity and extent of presynaptic endings of inhibitory neurons on various structures in the vertebrate nervous system are striking (Roberts 1980). The impression is gained that one is looking at a highly restrained nervous system, with the inhibitory neurons acting like reins that serve to keep the neuronal "horses" from running away. A major tenet of this hypothesis is that in behavioral sequences,

innate or learned, preprogramed circuits are released to function at varying rates and in various combinations. This is accomplished largely by the disinhibition of pacemaker neurons, whose activities are under the control of tonically active inhibitory command neurons, many of which may use GABA as a transmitter. According to this view, disinhibition is permissive, and excitatory input to pacemaker neurons would have mainly a modulatory role. Disinhibition, acting in conjunction with intrinsic pacemaker activity and often with modulatory excitatory input, appears to be one of the major organizing principles in nervous system function. For example, cortical and hippocampal pyramidal neurons literally are studded with endings from inhibitory GABAergic neurons. Not only are the endings of the GABAergic aspinous stellate neurons densely distributed around the somata and dendrites of the cortical pyramidal cells, but they also have been located on initial axon segments, where they may act as frequency filters. In addition, the GABA neurons have GABA endings impinging upon them. One then gets a picture consistent with the idea that the pyramidal cells are tightly inhibited by inhibitory neurons, which themselves may be inhibited by the actions of other inhibitory neurons or by cybernenes or hormones so that the inhibitory influence of some of them on the pyramidal neurons may be relieved. It has been shown that both inhibition and disinhibition play prominent roles in information processing in the cortex, hippocampus, and other neural regions (Roberts 1980).

Normally, the principal cells, which possess the capacity for spontaneous firing or great sensitivity to excitatory input, or both, may be held tightly in check by constant action of inhibitory neurons impinging on them. Information processing prior to the activation or release of the principal neurons may, to a considerable extent, be expressed via inhibition of the inhibitory nerves. Through disinhibition, neurons in one neural sector can fire at different rates and sequences and serve to release suitable circuits in other levels of the nervous system. Communication between neural stations and substations, I believe, takes place largely by throwing of disinhibitory neural "switches," and the activities within them take place largely through preprogramed, hard-wired circuitry. For example, this may be the way information flows from sense organ to cerebral sensory area, through associative areas to the motor cortex, and by way of the pyramidal paths to the final motor cells of the medulla and cord.

The Basic Neural Unit (Oversimplified and Partly Hypothetical)

Because I favor a disinhibitory model for normal nervous system function and disinhibitory mechanisms of neurological disorders, it is advisable to present some qualitative models of neural circuitry, not inconsistent with known neural arrangements, that might be useful in following the subsequent discussion.

Let us examine a simple linear series of three neurons and a muscle fiber. Neuron A controls through a single interneuron B the activity of a motoneuron C, which, if left alone, could discharge spontaneously at a rapid rate, causing the muscle fiber to contract. Let us also assume that interneuron B is a tonically active inhibitory neuron from which, in the absence of input from the first neuron in the series, A, an inhibitory transmitter is liberated at such a rate that the membrane potential of the motoneuron is held at a level below the firing level, and the muscle fiber does not contract. Neuron A is presumed to be a phasically active inhibitory neuron. When it is caused to discharge upon interneuron B, inhibition from the latter on motoneuron C will be decreased. As a result, the motoneuron will fire; this, in turn, will cause contraction of the muscle fiber.

Let us now substitute for the motoneuron and muscle fiber in the above model an entire simple neural circuit (figure 3). The principal or pacemaker neuron (P) for the operation of a particular neural unit would be analogous to the potentially spontaneously active motoneuron in the preceding model, and it would be under the restraint of the tonically active inhibitory interneuron, I_t. A phasically active inhibitory neuron, I_{p1}, when activated by excitatory afferent input impinging onto it from neighboring neuronal circuits or from circuits in other neural sectors, would inhibit I_t. This would make it possible for P to fire by relieving it of the tonic inhibition exerted by I_t, i.e., by disinhibiting it. Excitatory input also could help release the P neuron by direct depolarization, in some instances via dendritic spikes propagated electrotonically to the soma. In reality, there would be multiple excitatory and inhibitory inputs onto both the P and I_t neurons. A particular I_t neuron might inhibit a number of P neurons, as probably occurs in the case of hippocampal basket cells or retinal amacrine cells. However, the model requires that, in most instances, decrease or cessation of inhibitory signals from the I_t neuron would be a necessary, but not always sufficient, condition for the firing of the P

neuron. The latter might begin to fire spontaneously when partially or completely relieved of inhibition exerted by the I_t neuron, or excitatory input to the P neuron might be required for it to depolarize to the firing level, even when the tonic inhibitory influence on it has been decreased or completely removed. In the latter instance, less excitatory input would be required to fire the P neuron in the absence of inhibition by the I_t neuron than in its presence.

After an initial stimulus pattern is experienced by an organism, many neurons in various through-put sectors[1] of the CNS are either activated or inhibited. I propose that the consequence of effective afferent neural input to any sector from any other sector of the nervous system, starting with receptors, is the change in activity by combinations of disinhibition and excitation of groups of excitatory P neurons (figure 3).[2] The individual P neurons in these groups control through their activities through programs of activity in satellite neurons (S), excitatory interneurons, which in turn signal P neurons in other neural sectors and/or effectors (muscles, glands). The frequency of firing of the P neurons would at any given moment determine the exact details of such programs, since the presynaptic inpulses of the main axons invade axonal branches, the activities of which vary with the frequency of firing of the main axon. Frequency-dependent differential channeling of information can take place at points of axonal branching. Although I believe that the terminals of all of the branches of a particular neuron

[1]My concept of such a sector does not correspond exactly to a particular classically designated anatomic structure, but rather includes excitatory projection neurons and the excitatory interneurons upon which they impinge. For example, specialized thalamocortical cells and their excitatory cortical satellites (spiny stellates) are considered a "sector." In contrast to the through-put sectors, the projection neurons of analyzing sectors, such as Purkinje cells of the cerebellar cortex, frequently are inhibitory. The latter will not be discussed in detail here.

[2]Throughout this discussion, I have in mind cortical synaptic relationships. Thus, candidates for excitatory interneuronal satellite neurons (S) might be the cortical spiny stellates. Some nonspiny stellates may be the phasically active inhibitory (I_p) interneurons, and some nonspiny stellates and basket cells may be the tonically active inhibitory (I_t) neurons (Roberts 1980). The pyramidal neurons are presumed to be the projection neurons (P). Some pyramidal neurons from other sectors, possibly important largely for extremely rapid emergency communication, may synapse directly on P and I_p neurons without mediation of satellite cells. Some such inputs may go directly to satellite neurons (not shown in figure 6). The latter is most evidence in the case of the spinal motoneurons, which in primates receive direct input from pyramidal tract neurons. I recognize, of course, that details of circuitry differ from one neural region to another. I have omitted a neural element for feedforward inhibition in the unit illustrated in figure 3 in the absence of cogent evidence for its occurrence.

probably liberate only one true neurotransmitter, the effects of this liberation might be either excitatory or inhibitory, depending on the nature of the postsynaptic receptors that are affected by the particular transmitter. Recent data suggest that some neurons may contain a true transmitter and also a cybernene, i.e., GABA and one of several neuropeptides. Thus, the P neurons integrate the incoming signals at all levels, from dendritic endings to initial axon segment, and through their activities express frequency-dependent aspects of genetically coded neural programs that are carried out by the satellite neurons The excitatory satellite neurons of active circuits largely serve as the means of effective communication with other neural sectors.

Some phasically active inhibitory interneurons (I_{p2}) can furnish synaptic recurrent inhibition. These may be activated by recurrent collaterals of the P neurons and can exert hyperpolarizing postsynaptic inhibition on the same P neurons by which they are activated. Other inhibitory interneurons may be activated by collaterals of P neurons in the same sector (I_{p3}) or by collaterals of satellites activated by P neurons from other sectors (I_{p4}). Reflecting activity occurring in succeeding neural sectors, such collaterals would furnish the links for the feedback inhibition that is known to exist between neural sectors. Finally, there are presumed to be neurons (I_{p5}) that allow P neurons to inhibit neurons that are members of other modules in the same sector. I_p neurons also may exert presynaptic inhibition, wherever it exists (not shown in figure 3).

Neural Modules

Aggregates of mutually interrelated neurons, ranging from a few up to approximately 100 in number, are found in the cortex and elsewhere in the CNS and may serve as modules. I would like to speculate that in each neural sector, from receptor to cortex and cortex to effector, there are redundant, functionally similar (but not identical) groups of neural modules. Although the neurons within a given module, or even in several modules, may have had a clonal origin, unique aspects of location and experiential history could have given rise to a variety of structural and functional individual differences among them at any subsequent period of observation. At each functional level in the CNS, I presume there to be classes of modules that respond to given inputs that are first

determined at the level of receptor transduction and first-order neurons. In turn, these modules signal the frequency and intensity of input received by them in a manner interpretable by related modules in other sectors. Collaterals from their active P cells are presumed to impinge on inhibitory interneurons lying between the neural elements of different modules in such a manner that, after the onset of activity, each module would have a tendency to create an inhibitory surround in which adjacent or interpenetrating modules would tend to be held in a state of inactivity or reduced activity. An active cortical "column" could consist of vertically communicating modules and might be surrounded by inhibited "columns." Units for different or even antagonistic functions frequently might be located side by side, exerting an influence upon one another. Thus, the activities of modules subserving a particular function might inhibit some functionally redundant modules as well as some modules involved in parallel processing or in incompatible responses, such as flexion-extension, eating-satiety, waking-sleeping, warming-cooling, attacking-fleeing.

A module is postulated to have, among its various cellular components, groups of spatially distributed, functionally redundant P neurons and their associated S neurons, as shown in figure 3. Let us assume that as a result of existing patterns of connectivity, a particular class of impulses would begin to arrive largely from the satellites of P neurons of other sectors and that the two active P neurons shown in figure 3 would be potentially capable of responding to the arriving input. Excitation would take place via synapses directly on the P neurons, presumably largely on dendritic processes. Disinhibition is shown to occur by excitation of the phasically active inhibitory interneurons, I_{pl}, that act directly on the somata or proximal dendrites of the I_t neurons, the tonically active inhibitory neurons that synapse largely on the somata and initial axon segments of the P neurons. The P neurons are depicted as communicating with each other via axon collaterals that produce both excitation and disinhibition. Which P neurons in a particular module in a given sector would have their activities changed during the first submillisecond instant of arrival of a new input would be determined by their eligibility at the time, depending on factors such as the temporal sequences of their activation, their exact response characteristics, the degree of membrane polarization, and metabolic state. When partial or complete inactivation of the I_t neuron has taken place, the P neurons are released to fire and send impulses down their axonal

branches, resulting in the activation of the satellite neurons. Also, through their collaterals, the P neurons activate phasically active inhibitory interneurons, I_{p2}, which impinge back onto them and provide for inhibitory phasing of their own activity. The two active P neurons of a particular module in figure 3 are shown to inhibit their neighbor, presumed to be a member of another functional module, through an inhibitory interneuron (I_{p5}). Collateral branches of the P neurons also are presumed to produce inhibition in preceding neural sectors in the sequence, signaling to decrease or stop the input from those sectors. Such inhibition is indicated to occur mainly through the activation of a class of inhibitory neurons (I_{p4}) in the manner shown in figure 3 for excitatory inputs coming back from the succeeding sector to which the P neurons illustrated communicate through their satellites. The P neurons eventually may cease activity when the difference between the combined releasing effects of direct excitatory and disinhibitory influences and the inhibitory effects of the feedback influences and of the I_t neurons has fallen below a certain critical level. It is in the above way that I imagine communication between neural sectors to take place.

I suggest that with use, long-lasting connectivity increases might take place selectively at excitatory synapses on I_p neurons at the sites, synaptic and/or electrotonic, indicated by the boxes in figure 3. The origins of the inputs are indicated to be largely satellite neurons of P neurons from other sectors (e.g., the spiny stellate cells of the cerebral cortex that receive thalamic and/or callosal afferents or afferents from other cortical regions in the same hemisphere). The above formulation avoids the postulation of the occurrence of plastic changes taking place at synapses arising directly from, or impinging directly on, the P or I_t neurons. This would have the advantage of not tinkering with genetically determined "hard-wiring" of the nervous system and would preclude interference with basic communication codes that might exist between the I_t and P units. This formulation places the burden of modification by experience on synapses that are one or more synapses removed from the projection neurons in the mainline channels of communication. In figure 3, the P neurons in a given module activated by a given input are postulated to have mutually disinhibitory connectivities. Such connectivities would increase to a maximum with use within modules early during development. It is postulated that in the adult organism, the connectivities between units within a given module would undergo minimal change with

use or disuse. This also is suggested to be the case for inhibitory relations between different modules (synapses on I_{p5} neurons).

Most of the I_t and I_p neurons in the various regions of the vertebrate nervous system are presumed to employ GABA as transmitter, although glycine probably is an inhibitory transmitter in the cuneate nucleus and ventral regions of the cord and acetylcholine in the reticular nucleus of the thalamus and possibly along the whole neuroaxis. That at least some of the P cells and their excitatory satellites (not shown) may use glutamic acid, aspartic acid, or acetylcholine as transmitters is compatible with much indirect data, but is not proven. In the case of motoneurons, the excitatory transmitter is known to be acetylcholine.

Specialized Analyzing Regions

Nervous systems do not operate in a linear fashion. The strategy that appears actually to be employed by nervous systems is for P neurons in one neural sector to inform, directly or through their satellites, neurons in one or more other neural sectors about their activity. Minimally, a bifurcation of the flow of neural information from a particular sector takes place into direct through-put channels and also into those leading to analyzing regions such as the cerebellum, hippocampus, basal ganglia, the reticular nucleus of the thalamus, the association cortex, etc. In the specialized analyzers, information arriving from several sources is integrated, and the output reflecting this analysis, with variable time delays, then plays upon neural elements in the direct channels to adjust the activity therein so that it is more compatible temporally and spatially with activity elsewhere in the CNS in helping achieve adaptive responses to the environmental exigencies being faced at the time. The cerebellar cortex is one such region of analysis, and its output via inhibitory Purkinje cells may serve to adjust the phasing of various motor activities through its rich and powerful inhibitory output onto recipient cells in deep cerebellar and Dieter's nuclei. It is suggested that inhibition coming from cerebellar Purkinje cells, from cells projecting from the globus pallidus to the ventrolateral thalamus, neurons of the reticular nucleus of the thalamus to various thalamic nuclei, and from other analyzing sectors as well, creates a negative imprint on the flow of excitatory information in a manner similar to the punched holes in a player piano roll or a computer card (figure 4). The above also suggests

that there may be two basically different kinds of neural subsystems in the vertebrate CNS. There are those whose projection neurons are excitatory (the through-put systems) and those whose projection neurons largely are inhibitory (the analyzing systems). Without going into detail, current data suggest that a large proportion of the inhibitory interneurons in the CNS (e.g., the I_t and I_p neurons postulated in figure 3) and the inhibitory projection neurons from the various analyzing centers may use GABA as their neurotransmitter (Roberts 1980).

The Basic Neural Unit as a Switch and the Module as the Quantifier

We can consider the basic individual units within the modules, as postulated in figure 3, to be small switching neural networks or neural switches. When they are closed, paths are established between incoming and outgoing signals between neural sectors. Closure of a particular switch in a given module will increase the probability of the closure, or maintenance of the closed position, of other switches in the same module and decrease the probability of the closure of switches in other modules whose activation would lead to competing or unnecessarily redundant activities. Such increases or decreases in probabilities of closure of neural switches would be analogous to decreases or increases, respectively, in the resistances of springs in spring switches. The net depolarizing effect of the combination of disinhibition and excitation occurring at any particular moment on the P neurons depicted in figure 3 would be analogous to pressure applied to spring switches. When it is sufficiency great, contact is established, the circuit is completed, and information can flow from one neural sector to another. In the biological situation, many metabolic, hormonal, and microenvironmental factors could affect the probabilities of neural switch closure. For example, the endogenous release or exogenous administration of an agent that decreases the efficacy of the tonically inhibitory interneurons (I_t in figure 3) would decrease neural inhibition and would, by the analogy I am using, decrease the resistance of the springs in the neural switches. Then many more neural switches might be closed and pathways opened by a given input than would otherwise occur. Since many of the I_t neurons might restrain the activities of P neurons by tonic activity and liberation of the inhibitory neurotransmitter GABA, decreases in

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the rate of release or exogenous administration of substances that block the postsynaptic action of GABA or desensitize postsynaptic receptor sites would increase the ease with which neural switches would be closed and circuits activated. The endogenous opioids, the enkephalins, markedly decrease the efficacy of a number of GABAergic pathways in the vertebrate nervous system and have been shown specifically to depress inhibitory interneurons in the hippocampus, thereby increasing the firing rates of the pyramidal cells through disinhibition (Nicoll et al. 1980). On the other hand, the release of endogenous enhancers of GABA action or the administration of substances such as diazepam, some barbiturates, diphenylhydantoin, and valproic acid seems to amplify the efficacy of GABA at postsynaptic sites and have the opposite effects. Some of the latter and related substances and local anesthetics also can increase the resistance of the neural switches by decreasing the conductile properties of the P neurons themselves (Roberts 1980). Thus, in the analogy I have made between neural and mechanical spring switches, the resistance of the springs can vary from moment to moment, depending on internal conditions, and it can be influenced by many endogenous factors and by the exogenous administration of drugs.

Consequences of Incoordination Between Inhibition and Excitation

Extensive analyses of the locations and functions of inhibitory GABAergic neurons make it feasible to consider the vertebrate central nervous system as a highly restrained one. GABAergic neurons are important for the control of activity in all parts of the vertebrate CNS (Krogsgaard-Larsen et al. 1979; Roberts 1976, 1977, 1980; Roberts et al. 1976). Derangements in function of these neurons may be involved in various forms of epilepsy, in Huntington's and Parkinson's diseases, in schizophrenia, and possibly in the genesis of brainstem- and hypothalamus-related "psychosomatic disorders." There is support for the view that disinhibition may be one of the major principles involved in nervous system function. *The major tenet of this hypothesis is that in behavioral sequences, innate or learned, preprogramed circuits are released to function at varying rates and in various combinations largely by disinhibition of pacemaker neurons whose activities are under the control of*

tonically active inhibitory command neurons, many of which use GABA as a transmitter. According to this view, disinhibition is permissive, and excitatory input to pacemaker neurons would have largely a modulatory role. Disinhibition, acting in conjunction with intrinsic pacemaker activity and often with modulatory excitatory activity input, is one of the major modes of nervous system function.

The successful or adaptive operation of a nervous system requires a coordination of neuronal activity in order to prevent too-frequent or too-infrequent firing of preprogramed neural circuits. When gross malfunctions of the coordination of inhibitory and disinhibitory neuronal systems or between the neuronal and cybernene systems occur, lethal effects may result either through generalized seizures or cessation of operation of some vital function; alternatively, some obviously severe neurologic-physiologic dysfunctions may occur.

When there is persistent incoordination between the GABA system and other neurotransmitter and cybernene systems, for whatever reason, the defect might be restricted to a local brain region, might include several regions, or might be global throughout the CNS. A critically placed local incoordination may have drastic reverberations in the whole nervous system, as seen in grand mal seizures arising from focal cortical lesions. Under relatively simple environmental conditions, the nervous system in such individuals could function in an apparently adequately adaptive manner, which might appear to be in the normal range. As the complexity and intensity of environmental inputs are increased, there would be a correlated increased degree of incoordination. Then those subsystems in the nervous system that are most poorly controlled will tend to break down under the stress and produce symptoms that are consequent with such a breakdown.

Let us suppose that for some reason, in the entire brain or in specific regions, tonically active inhibitory GABA neurons have considerably lower than normal effectiveness on their recipient neurons, which themselves are normally effective; or, alternatively, that the GABA neurons are normal and the neurons they ordinarily control are less than normally responsive to GABA or are potentially hyperactive because of intrinsically arising or extrinsically produced membrane changes. With increasing excitatory input, there would be an increased tendency to release pacemaker neurons. More than normal numbers of behavioral options or inappropriate ones (e.g., behavior disorders, mania, schizophrenia)

would be released; choreic movements, seizures, or spasticity might occur; there might be hypersensitivity to visual, auditory, tactile, olfactory, gustatory, or pain stimuli. GABA neurons play important roles in control mechanisms in various hypothalamic and brainstem centers. Thus, if specific regions within these latter structures were affected, greater than normal degrees of changes in responses might be observed, for example, in emotional reactivity, cardiac and respiratory functions, blood pressure, food and water intake, sweating, galvanic skin response, insulin secretion, liberation of gastric acid, motility of the colon, etc.

Under a variety of environmental circumstances, both internal and external, an organism must maintain within physiological limits the rates of operation of continuously needed neuronal circuits, such as those required for cardiac function, maintenance of blood pressure, and respiration. This requires a delicate balance between inhibitory and disinhibitory neuronal systems and within and between the neuronal and neuromodulatory elements in brain regions controlling these functions. A critically placed persistent incoordination in pertinent brain regions eventually may lead to peripheral pathologies. For example, it may be conjectured that many cardiovascular disorders have their origins in the brain—a concept not foreign to those concerned with so-called psychosomatic or stress-related diseases (Roberts and Krause in press). The nervous system regulates the circulation to different vascular beds with a high degree of specificity, provides a range of coordinated responses that are appropriate to the metabolic needs, and couples cardiovascular changes with other autonomic and somatic activities to produce an integrated response (Calaresu et al. 1975). Recent experiments have begun to delineate the key roles of GABA neurons in control of both central sympathetic and parasympathetic outflows to the heart and vasculature and in the coordination between them. Incoordinations in these regions resulting from inadequate function of GABA neurons could lead to cardiac arrhythmias and large fluctuations in blood pressure that would predispose to cardiac, vascular, and cerebral damage (DiMicco et al. 1979; Gillis et al. 1980; Williford et al. 1979). In schizophrenia and in Huntington's and Parkinson's diseases, as well as in other disorders such as the dementias, there may be defects in the structural or functional relationships of GABA neurons with other neurons, which either might inhibit or excite them or which they themselves inhibit.

Individual and Group Behavior in Neural Modules: Key Physiological Actions of Alcohol

I believe that the permissive element in the activity of the P neurons (see figure 3) is the release from inhibition by the tonically active I_t neurons and that the excitatory input directly onto the P neurons generally might not be sufficient by itself to release the P neurons for activity during normal nervous system function. As the intensity of a particular input is increased from some low basal level, there would be a corresponding increase of excitatory influences on the I_p neurons and on the P neurons themselves, resulting in release of a correspondingly greater number of P neurons. A point could be reached at which one of the P cells in the sector that is no longer capable of being restrained by its I_t neuron would begin to fire spontaneously, occasionally or in a rhythmic fashion characteristic for that neuron. The input from the excitatory collaterals of this neuron could add to the excitatory and disinhibitory influences on P neurons in the same module, recruiting them into synchronized discharge patterns (Johnston and Brown 1981). This could continue until virtually all available P neurons in the modules of a given sector are involved in responding to a pertinent input. Increases in connectivity could occur at some synapses *between* modules in a given sector, thus facilitating their recruitment by each other in future rounds of activity. In this way, I envision that the neural oscillators discussed previously could be activated.

Synaptic recruitment within modules probably occurs largely through pathways involving axon collaterals. Recently, strong evidence has been adduced for the presence of recurrent excitation between hippocampal pyramidal cells in the CA3 region (MacVicar and Dudek 1979). Spikes in one pyramidal cell can evoke spikes in another pyramidal cell in keeping with the suggestion in figure 3. *In considering the firing of pyramidal neurons and their synchrony in discharge, one also must give heed to the possibility of electrotonic interactions among them through gap junctions.* Gap junctions, between dendrites, somata, or axons of adjacent neurons, may be important in complex information processing in the vertebrate CNS (Bennett and Goodenough 1978; Gutnick and Prince 1980; Llinás 1975; MacVicar and Dudek 1979, 1980). There may be conditions under which a relatively small number of released pyramidal cells may, through such contacts, help enlist the

cooperative firing of other pyramidal neurons. Recent experiments with fluorescent dye injection indicate that hippocampal (MacVicar and Dudek 1980) and cortical (Gutnick and Prince 1980) pyramidal cells may be coupled to other pyramidal cells through electrotonic junctions. Thus, electrotonic coupling combined with recurrent excitation could account for the synchrony and spread of paroxysmal discharges in these structures. A possibility also exists that the formation and dissolution of electrotonic junctions between neuronal processes may be occurring continuously and that alterations of environmental factors, e.g., membrane potential, pH, or extracellular levels of calcium ions, may play a role in determining the extent and stability of such junctions at any time (Bennett and Goodenough 1978).

There are several ways by which enhancement of synchronic activity may arise—specific increases in rate of release of synaptic excitatory transmitters; blockade or desensitization of inhibitory transmitter receptor mechanisms; decreases in the availability of inhibitory transmitter itself; decreases in activity of inhibitory neurons; and increases in the ease with which electrotonic junctions are formed or activated. There may be specific decreases in the rates of formation or release of inhibitory transmitter because of either decreased function or actual destruction of the inhibitory neurons. A model applicable to such altered interactions within a population of affected cells is given in figure 5. The experimental observations relevant to one or another of the above mechanisms are legion (Roberts 1980). I will concern myself here only with the items related to the effects of alcohol on increasing the probability that neural modules in key brainstem regions will operate in the mode shown in figure 5, rather than that in figure 3. The general aspects of the relationship of the model to seizures have been discussed in detail (Roberts 1980). Seizure-like activity in some low-threshold brainstem regions may signal approach to the hedonic state, while in the motor cortex it may give rise to convulsions and, in the hippocampus, to amnesia.

I believe that the synchronic activities in the so-called reward centers that are associated with behavioral addiction to alcohol and to other drugs and to some purely existential aspects of the environment may be akin in principle to local seizures in specific low-threshold subcortical centers. The plastic changes that take place during addictive processes may sensitize these neural substrates to paroxysmal discharges when the particular agent or set of circumstances is present. The case of association with the purely

environmental signal is simpler to understand, e.g., experience of mother sets off the food- and drink-associated reward circuits in a hungry infant. In the adult, many symbolic equivalents may do the same; witness the high value placed on the "soul foods" mother used to make. Alcohol and probably other drugs may make the circuits easier to fire, *and* a variety of circumstances associated with their firing when alcohol is present may become firmly linked with releasing them. Thus, the smell or sight of an alcoholic beverage or its container, a particular bar or bartender, or a host of other more subtle internalized signals may set off the appropriate anticipatory circuits, compelling an individual to the consummatory act of drinking an alcoholic beverage. In other words, alcohol and other addictive drugs may "sensitize the neural substrate involved with natural reward" (Kornetsky and Esposito 1979). Metaphorically, these substances may be considered to grease the behavioral slide as well as to furnish the impetus to go down it. In this regard, it is instructive to observe individuals who have plenty of alcohol at home anxiously waiting for their favorite bar to open to obtain their first drink of the day.

From the foregoing discussion, it may be suggested that "the electrotonic synapses may change the dynamic properties of cellular ensembles in order to generate synchronous neuronal firing" (Llinás 1975). Inhibitory shunting may be the basis for dynamic uncoupling (Spira et al. 1976). Looking again at the module in figure 3, it is apparent that with asynchronous synaptic inputs coming into the individual P cells and with accompanying asynchronous activity of the inhibitory (I) neurons, the P cells would tend to fire as individuals, communicating with each other largely synaptically. A number of changes in the mode of operation might tend to swing the balance toward synchronic, electrotonically generated activity in the module. A general decrease in all synaptic activity, excitatory and inhibitory, would unquestionably favor the latter mode of activity. Of a large number of effects of alcohol that have been reported, all probably stemming from initial effects on membrane fluidization (Johnson, Friedman, Cooke, and Lee 1980; Johnson, Lee, Cooke, and Loh 1980), the most pertinent at this level of analysis are the reported decreases in the releases of several neurotransmitters (Carmichael and Israel 1975; Erickson and Graham 1973; Faber and Klee 1976; Sinclair and Lo 1978; Subramanian et al. 1978). One of the earliest neural effects of alcohol apparently is a presynaptic impairment of neurotransmitter release. If, in addition to the latter, alcohol would facilitate in

some manner the functionality of gap junctions, the conditions would be favorable for alcohol, at levels below the anesthetic one, to help set into action oscillatory behavior of neural modules in different regions in the CNS. This would be likely to occur first in the sensitive, delicately poised ones likely to be associated with various aspects of consummatory behavior, especially in those regions with little or no blood-brain barrier, such as the hypothalamus. The latter effects also would tend to uncouple the operation of the oscillators from the stimuli normally associated with their activity and to link them with the presence of alcohol and with the circumstances associated with its intake. The following is particularly pertinent to the latter point. Intracellular recordings from electrotonically coupled neurons in the isolated visceral ganglion of the snail *Planorbis corbeus* showed that alcohol induced bursting among them (Pentreath and Berry 1978). The following quotations from Pentreath and Berry (1979) are of interest:

> The electrically coupled neurons normally fired action potentials spontaneously at a fairly regular rate, but with a tendency to produce couplets of spikes. Some fired in synchrony, other independently, depending to some extent on the strength of coupling. Ethanol (0.6-1 percent) caused a marked change in this firing pattern. Recurrent hyperpolarizing potentials and alternating bursts of action potentials occurred progressively until all the neurons were actively and rhythmically bursting in synchrony. These effects were produced within 1 min of application, were maintained as long as ethanol was present (tested up to 2 h) and were reversed within 2 to 3 min of washing with pure saline.

> The bursting elicited by ethanol can be distinguished from that which sometimes occurs spontaneously and which can be elicited when one of the neurons is depolarized by continuously applied current. The latter appears to be caused by regenerative excitation, and synchrony of spikes occurs only at the end of each burst. . . . During bursting induced by ethanol, all the neurons fire more or less in synchrony throughout the burst. . . . Occasionally, preparations exhibited natural bursting activity similar to that induced by ethanol. Thus, it appears that ethanol is "switching on" a process which may occur normally. In neurons showing spontaneous bursting, ethanol increased the amplitude of postburst hyperpolarization and the number of spikes per burst.

The possibility was suggested "that the initiation of bursting results from ethanol-induced changes at the electrical junctions between the neurons."

It has been known for some time that in mammals electrotonic coupling exists among neurons in the inferior olive (Llinás and Volkind 1973). Recent experiments have shown that a single injection of alcohol in rats results in a dramatic increase in the frequency of bursts of activity from the electrotonically coupled

neurons in this structure when monitored by extracellular recording of activity in the Purkinje cells of the cerebellar cortex that receive their input (Rogers et al. 1980). These effects resemble those of harmaline, a drug that also produces strong synchronous and rhythmic activity in the inferior olive (DeMontigny and Lamarre 1973; Lamarre and Mercier 1971; Lamarre et al. 1971; Llinás and Volkind 1973; Sjolund et al. 1977), possibly acting similarly to alcohol by generally decreasing presynaptic release of the neurotransmitters involved in neural inputs to this region (Javoy et al. 1977; Kim et al. 1970; Sjolund 1977; Sokolove and Roth 1978; Vargas and Erlij 1976). It has been suggested that the synchronous activity is dependent on the degree of electrotonic coupling between the dendrites of the olivary neurons and that the activities of inhibitory interneurons regulate the extent of coupling (Llinás 1975). I would like to suggest that even though both harmaline and alcohol may decrease the release of a variety of transmitters from nerve terminals, the key event in the effects of these substances on the inferior olive may be the decrease in release of GABA from terminals of GABAergic neurons, converting the situation from that depicted in figure 3 to that of figure 5.

There is hope that eventually the effects of alcohol and other drugs on electrotonic interactions among neurons will be understood at the molecular level. Great progress is being made in the understanding of the nature of gap junctions, which probably furnish the major modes of cell-to-cell communication (Finbow et al. 1980; Gilula et al. 1972; Hertsberg and Gilula 1979; Loewenstein 1979; Peracchia 1980; Revel et al. 1980; Yancey et al. 1979). The major protein components of gap junctions of liver and lens have been isolated and are being studied in great detail. It already appears that the structures of these largely hydrophobic proteins from a particular tissue are conserved between species, but that the gap junction proteins of different tissues from the same species show great differences (Takemoto et al. 1981). One awaits eagerly the isolation and characterization of gap junction proteins of tissue of the nervous system, because understanding of how these proteins function in lipidic membrane environments may elucidate the nature of some of the effects of alcohol and other drugs as well as how the relative proportions of synaptic and electrotonic communications occurring at a given time may be regulated when no exogenous substances are present.

A Scenario for Alcoholism

Awed by the multidimensionality of the vast and intimidating panorama of the effects of alcohol, I am reduced to imagining a scenario for the concatenation of occurrences that might lead to the sequential changes from an occasional drink to frank alcohol addiction. The portion of the following discussion in italics is an imagined case history; the rest is my commentary on it.

Let us start with the effects at a cocktail party of a single alcoholic beverage, or two, drunk relatively rapidly on an essentially empty stomach by an individual in a pleasant social setting. Shortly after the consumption of the drinks, there is an exhilaration, a rush of good feeling in which the cares of the day seem more distant than they were immediately before, and various aspects of the ambient environment, physical and social, more pleasant than before the drink. A behavioral disinhibitory effect may be noted in which speech becomes louder and possibly more fluent, appetite is sharpened and consumption of available food increases, interest in potential sexual partners is heightened, and an increase in the extent and smoothness of movement occurs. Thus, as with a variety of potentially anesthetic substances, there is an early disinhibitory behavioral phase during the rapid rise of blood alcohol levels. It often is said at this point, "I am beginning to feel no pain." In terms of the model in figure 1, it might be said that the tendencies to displace the individual from the equilibrium or hedonic state, such as anxieties about unresolved issues of the day's activities, have been decreased. Anticipatory, reward-related consummatory circuits have become activated, and facilitation has occurred of some of the behavioral options that are sensed to be leading to achievement of the equilibrium state. Under the latter conditions, there begins to be a dissociation between the realities of the surroundings and the inner experience of the individual, the causal links between the two becoming weakened, although it rarely is it realized that this is the case. Food that ordinarily may be eaten sparsely, if at all, becomes attractive and may be consumed in great quantities. This is epitomized in a television commercial showing a man taking an antacid preparation on the morning after a party while saying, agonizingly, "I ate too much, I ate too fast." People may seem more attractive and friendly than before, and conversations may begin between individuals previously too shy to communicate. Exaggerated statements, inappropriate social arrangements, and unsolicited

sexual advances may be made that may be regretted and for which apologies may be made subsequently under conditions of sobriety. Whatever generally is occurring during the "happy hour" tends to be learned rapidly and to be remembered in the context of the feelings that are engendered. Thus, when alcohol rises rapidly to certain levels in the blood and tissues, I propose that hypothalamic and brainstem consummatory-related reward neural systems are more easily released to oscillate synchronously and, by their oscillations, to communicate "pleasure" to limbic and cortical structures and to insure its remembrance (Kornetsky and Esposito 1979).

At the reward level, the aboriginal events are believed to be related to the membrane-fluidizing effects of alcohol after rapid entry into tissues from the blood. Of the many possible effects of alcohol, when given in intermittent, potentially addictive doses, I have presumed one of the most important to be the decreases that it causes in the presynaptic release of various neurotransmitters. Perhaps the greatest effect of a single dose of alcohol would be a decrease in the ability of the sensitive, tonically active GABA neurons to hold the neurons of the oscillators in check; but decreases would also occur in the amounts of excitatory transmitters liberated by neurons in primary afferent pathways and their relay stations. The above, together with possible direct facilitation of electrotonic communication via gap junctions resulting from the membrane-fluidizing effects of alcohol, would favor the occurrence of the synchronous oscillatory behavior of the pertinent neural groups. Because of paroxysmal discharges of the latter, it would be expected that release of transmitters from the terminals of their neurons would be greatly increased at their recipient sites over that found when the neural components of these circuits are operating on an individual basis, even though the presence of alcohol would be expected to decrease transmitter release below that occurring if the synchronic discharges occurred in its absence, such as if it were caused by electrical stimulation or by natural consummatory situations.

In addition to direct alcohol-induced decrements in numbers and intensities of externally and internally generated stimuli because of decrease in release of excitatory neurotransmitters from the primary afferents and their related circuits, there also would be increased inhibitory feedbacks from the discharging consummatory-reward circuitry. This is illustrated in figure 3 by the feedbacks exerted on the P neurons through the activation of inhibitory I_{p4}

neurons. Herein may lie the neural basis for the dissociation between inner experience and external realities that can occur with alcohol. This may also be the basis, under normal conditions, for the relative nondistractability of organisms when about to perform consummatory responses and when the responses actually are taking place.

It now is necessary to be more specific about the brainstem systems postulated to communicate the experience of pleasure and to insure remembrance of it and of the ambience in which it occurs. In the absence of truly definitive data, I can make my nominations only as one would nominate candidates for a primary election. The catecholamine, dopamine, and norepinephrine systems (Crow 1979; Hoebel 1979; Routtenberg 1979) and the endorphins (Stein and Belluzzi 1979) have been implicated in the operation of the reward centers. The serotonergic neurons of the Raphé nuclei also cannot be left out of the picture. I believe that it is likely that cholinergic neurons may play a key role in the memorial part of the process. A large body of physiological, chemical, histochemical, and immunocytochemical evidence supports the idea that there are a number of separate neuronal groupings in brainstem regions that specialize in the release of the above substances.

Much has been written over the years about the catecholamine and indoleamine systems and, more recently, about the endorphins, but the subject of the cholinergic neurons is less well known to the general scientific public. Although cholinergic neurons unquestionably play important roles in information processing in the CNS, limitations in our thinking about their precise participation still exist because of the inadequacy of the techniques available for the precise visualization of their somata and processes. Immunocytochemical studies are just beginning that are using antisera to the acetylcholine (ACh) biosynthetic enzyme, cholineacetyltransferase (CAT), which is presumed to be the most specific marker for cholinergic neurons (Kimura et al. 1980). Nonetheless, much data suggest that many of the effects of cholinergic neurons in the CNS, other than those ascribable to spinal motoneurons and their collaterals, are inhibitory on postsynaptic membranes (see Houser et al. 1980 and Wenk et al. 1980 for citations). In each neural sector there are varying proportions of cholinergic local interneurons and terminals of cholinergic projection neurons. Biochemical measurements of ACh contents and CAT and acetylcholinesterase (AChE) activities before and after suitable lesions showed clearly that in the striatum almost all of the cholinergic elements must be

attributable to neurons indigenous to this structure (Wenk et al. 1980).

The striatum is not a through-put sector but rather an analyzing region (see figure 4 and related discussion), a portion of whose projection fibers appears to arise from GABAergic neurons whose somata lie within it (Ribak et al. 1979). On the other hand, another analyzing region, the reticular nucleus of the thalamus, in which GABA neurons sending inhibitory projections into the thalamus are the major cell type, receives a large extrinsic inhibitory projection of cholinergic terminals from cells in the mesencephalic reticular formation (Houser et al. 1980; Wenk et al. 1980). The latter input probably inhibits the GABAergic neurons of the thalamic reticular nucleus and thus disinhibits or facilitates the flow of thalamic information to the cortex. The following statement (Scheibel 1980), written prior to our knowledge of the above transmitter relationships, expresses cogently and eloquently the potential importance of the above system:

> From these data, the concept emerges of a reticularis complex selectively gating interaction between specific thalamic nuclei and cerebral cortex under the opposed but complementary control of the brainstem reticular core and the frontal granular cortex. In addition, the gate is highly selective; thus, depending on the nature of the alerting stimulus or locus of central excitation, only that portion of nucleus reticularis will open which controls the appropriate subjacent thalamic sensory field. The reticularis gate becomes a mosaic of gatelets, each tied to some specific receptive field zone or species of input. Each is under delicate yet opposed control of (a) the specifically signatured sensory input and its integrated feedback from sensorimotor cortex, (b) the mesencephalic reticular core with its concern more for novelty (danger?) than for specific details of the input experience, and (c) the frontal granular cortex-medial thalamic system more attuned to upper level strategies of the organism, whether based on drive mechanisms (food, sex) or on still more derivative phenomena (curiosity, altruism). Perhaps here resides the structurofunctional substrate for selective awareness and, in the delicacy and complexity of its connections, our source of knowing, and of knowing that we know.

Obviously gross disturbances produced by alcohol in the function of the reticular nucleus of the thalamus by potently inhibiting its GABAergic neurons could lead to aberrations of perception and behavior because of a great and chaotic input into the cortex. However, smaller amounts might have salutary effects by helping achieve an optimal adjustment of thalamic gatelets, as well as possibly other such gatelets in the CNS, that are too tightly closed by the inhibitory action of GABA neurons. I have learned that members of a perennially victorious Olympic sharpshooting team

regularly take a sip of a strong alcoholic beverage (cognac) from a tube attached to a plastic pinch bottle hidden under their jackets just prior to taking their position before the target!

Figure 6 (taken from Scheibel 1980) shows a schematization of the ascending and descending projections of a single reticular neuron in the nucleus gigantocellularis of the basal forebrain, which I presume to be cholinergic. Figure 7 (taken from Wenk et al. 1980) shows a schematic summary of the direct monosynaptic cholinergic pathways from magnocellular nuclei of the basal forebrain of the rat to the cortex. These neurons, whose branches fan out in a manner somewhat similar to that observed for the brainstem noradrenergic and dopaminergic neurons, are seen to terminate in various cortical and subcortical loci, often after long traverses away from their cell bodies.

Acetylcholine, released synaptically simultaneously with ATP, may regulate the phosphorylation of membrane proteins by the ATP (Gordon et al. 1980). The postsynaptic transmembrane flux produced by interaction of ACh with its receptor may trigger cascades of reactions with major effects on macromolecular synthesis in the postsynaptic cell (Gisiger 1971). It is clear that trophic influences of nerves that are distinct from their roles in impulse conduction and transmission may be mediated by specific proteins that are transported from presynaptic to postsynaptic structures (Markelonis et al. 1980; Oh and Markelonis 1978). The activities of brainstem cholinergic neurons, through release of acetylcholine and trophic factors from their terminals, may be important in the plastic changes involved in memory. It is of interest in this connection that the anticholinergic drug scopolamine has strong amnestic effects in humans (Drachman 1977).

I would like to propose at this point that under normal conditions, asynchronously active elements of the brainstem cholinergic and catecholaminergic systems are involved in an important way in establishing a state of readiness in the CNS when the organism perceives that a problem exists with regard to need satisfaction or survival. When a solution to the problem becomes apparent, the latter systems are released from inhibition largely exerted upon them by tonically active GABA neurons (I_t neurons, figure 3). Inhibition of such GABA neurons may be achieved, at least in part, by the action of neurally released endogenous opioids, the enkephalins, which are known to markedly decrease the efficacy of a number of GABAergic pathways in the vertebrate nervous system and have been shown specifically to depress

inhibitory interneurons in the hippocampus, thereby increasing the firing rates of the pyramidal cells through disinhibition (Nicoll et al. 1980). When disinhibited, the cholinergic and catecholaminergic neuronal groups individually discharge synchronously in an oscillatory fashion, and the several neuronal groups active in this manner in the brainstem become phase-locked with each other and, eventually, with communicating groups in the other neural sectors. The paroxysmal discharges of the catecholaminergic groups give rise to the effect associated with reward (pleasure or a "high"). Those of the cholinergic system set up the conditions that make it possible for those plastic changes at neuronal junctions that are related to the establishment of memory eventually to take place.

At this point, however, the memorial system is on "hold" until some further substances or conditions appear, still unknown, that signal that the problem the organism is facing is being solved. The synchronic discharges of various neuronal groups throughout the nervous system are anticipatory of the release of response options, inherited or learned, that are likely to be effective in solving the problem; the closer the approach to a solution, the greater will be the synchrony of the participating neuronal groups. Feedbacks from the bursting circuits block a large portion of the sensory input at the moment of response, so that the behaviors decided upon may be released like rockets from a launching pad, with minimal interference. Soon after the release of behavioral responses, if internal and external signals indicate that the target has been hit, namely, that food or sex has been attained or escape achieved (or their symbolic equivalents), then the particular substances may be released or conditions achieved that allow delocalized plastic changes to take place that associate elements of the circuitry active at the time in all parts of the CNS. This occurs coincidentally or just after the desynchronization of the circuits whose coherent activity had served as the "motivation" for the behavior.

The individual we observed at the cocktail party in the previous instance now has become popular in his circle and, for business or professional reasons, feels obligated to attend such occasions several times a week. Lunch dates at which one or more drinks are consumed also become more frequent. A cocktail before dinner at home has been a custom for some time. The pressures and anxieties of work and home are somewhat relieved each time a drink is taken. Soon our hypothetical subject begins to look forward to each opportunity for alcohol consumption and even begins to keep a bottle in his desk drawer at work from which to take a drink when the

interval between such opportunities seems too long. He now needs more alcohol than before to get the desired effect. He has become an alcoholic. When he drinks too much he gets depressed. When he waits too long between drinks he gets fidgety, irritable, and overexcited. If he titrates himself carefully, he can function normally and even seem sober to family and associates, providing he sucks on an odor-killing lozenge. He has tried to break himself of his alcoholic habit by exerting will power, through individual counseling and in group therapy, by attending church, and by joining Alcoholics Anonymous. Each time, a period of abstinence has inevitably been followed by a return to drink. During abstinence he ate more, was irascible, and his ability to concentrate was impaired.

From the foregoing, I believe that the addictive effects of alcohol are related to the ingestion of single effective doses, at regular or irregular intervals, which give rise to rapid elevations in blood levels followed by rapidly falling ones. The aboriginal events are believed to be related to the membrane-fluidizing effects of alcohol after rapid entry into tissues (Chin and Goldstein 1977; Curran and Seeman 1977; Johnson, Friedman, Cooke, and Lee 1980; Johnson, Lee, Cooke, and Loh 1980). There may follow decreases in release of a variety of neurotransmitters from their nerve terminals, the key one possibly being that of GABA, and the facilitation of operation of gap junctions (Finbow et al. 1980; Takemoto et al. 1981). This may lead, in turn, to the oscillatory discharges of euphorigenic neural circuits whose release ordinarily is coupled to anticipation of solutions of problems related to consummatory activities. The discharge of these circuits in the presence of alcohol, and possibly other addictive drugs, becomes rapidly and firmly associated in conditioning paradigms with the alcohol itself and with the environment in which this occurs, so that eventually the particular setting, portions of it, or even reminders of it literally may "drive" an individual to drink.

Current data suggest that as a result of a concatenation of physiological changes that take place in nervous system function, eventually an individual addicted to alcohol may drink to prevent withdrawal, that is, to maintain a given blood level of alcohol. A person can do this in a social setting or in isolation, tending to drink more in a social setting or at work than when alone, and when exercising than when at rest. Even watching exciting athletic events on television may lead to increases in alcohol consumption.

In the absence of definitive data, it may be suggested that the metabolic consequences of stresses of all kinds may lead to

increases in the rate of oxidation and/or excretion of alcohol and, therefore, its removal from blood and tissues. The consequent lowering of blood and tissue alcohol levels may lead to increased intake so as to prevent withdrawal symptoms (see Schachter 1978 for an elegant, potentially applicable experimental design for testing this hypothesis). Such an individual may become an alcoholostat, just as a heavy smoker appears to be a nicotinostat (Schachter 1978). I believe that the hypothesis that an established alcoholic drinks to maintain a given blood alcohol level should be tested prior to accepting more elaborate psychological hypotheses.

In the human situation, genetic factors influencing rates of metabolism of alcohol and acetaldehyde and psychologically complex and variable events may be associated with the original addiction to alcohol, and the details of the behavior of one alcoholic may differ greatly from that of another. Nonetheless, the basic principles underlying the phenomena of addiction, tolerance, and withdrawal may be the same in human beings as in the several species of animals in which addiction can be produced experimentally. Some of the techniques reported to have been employed by traders in addicting the susceptible American Indians to alcohol on a large scale were not unlike those used today to addict susceptible strains of rats and mice.

Once alcohol becomes the predictable reinforcer, attempts to "cure" the condition by reasoning with the individual are notoriously ineffective because cortical centers associated with the processing of verbal information apparently do not exert major controls on the basic consummatory circuits. Rather, they seem to be employed to rationalize the attainment of the consummatory needs signaled from below. It seems as though effective behavioral treatment requires the complete substitution for alcohol with something or someone that is approximately as effective as alcohol for a period of time adequate for the restoration to normal of the physiological alterations that are related to the phenomena of tolerance and withdrawal (see below). In a preceding section of this paper, I proposed the intimate, primal relationship of the mothering person to the operation of the reward-consummatory circuitry. If the above conjecture is correct, then whatever evokes the experience of mother should have some efficacy in the treatment of alcoholism and other addictions as well. Envelopment in a caring group, such as Alcoholics Anonymous, can have important mothering aspects to it. However, removal from such a group may result in ready recidivism unless an adequate internalization of experience

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with it occurs, which is difficult in adult life. Sometimes, association with an exceptionally caring and mothering individual such as husband, wife, or psychiatrist may be effective, but this also often suffers from a continuing need for contiguity. Some religious and political groups that have mothering qualities have added props to their armamentarium. These include rituals, frequent rote repetitions of dogma, rigid dietary restrictions, and beliefs in a central mothering figure—often a living or mythic male figure with some overt or implied feminine characteristics. These latter approaches may be effective in substituting a behavioral addiction for a chemical one, but may lead to a stereotypically rigid existence, deviation from which causes intense anxiety and may lead to return to alcohol or some other addicting drug. The difficulties involved in achieving freedom from such situations are epitomized in daily reports of experiences of those who attempt to "wean" members of various cult groups away from them and back into the mainstream of everyday life. Conditioned aversion has been attempted, but, in my opinion, such procedures generally have been more therapeutic for those who feel a hostility toward alcoholics than in the treatment of the condition itself.

From the above, one must conclude that prophylactic measures would be far more desirable than therapeutic ones and that they must be diligently sought. The structures of modern societies have such large inertial elements in them that it is unlikely that changes at the societal level should be proposed, since their achievement is highly unlikely. Furthermore, no one is sufficiently cognizant of the key social variables that should be manipulated so as to alleviate the situation for susceptible individuals. We now must ask what the chemist, physiologist, or pharmacologist can do.

Let us look at the possible sequence of events in our hypothetical case history in somewhat more detail and during the process attempt to suggest bases for potential prophylactic and therapeutic measures at each stage.

1. Even with brief acute exposure to relatively low levels, alcohol can parasitize the reward circuitry of the brain and become a predictable reinforcer, just as lever pressing becomes when it results in electrical activation of the same brainstem centers. If this could be prevented, addiction to alcohol presumably would not take place. In the human situation, the only strategy that currently seems feasible to achieve this goal is to attempt to devise tasteless, soluble, and nontoxic substances that when added to alcoholic beverages

would allow them to retain their euphorigenic effects while attenuating their addictive properties. I propose this because, if the former effects were eliminated, it is probable that such effects would be sought through the use of other addictive drugs. It has been postulated in a preceding section that the brainstem catecholamine systems may be most importantly involved with the euphorigenic effect, while the cholinergic system may be more related to the plastic changes involving remembrance of the alcoholic experience, although these systems are certainly mutually interactive at all times and have complex relations with all other aspects of nervous system function. From this it would be predicted that presence in the alcohol of appropriate amounts of cholinergic-blocking agents, such as scopolamine or atropine, might attenuate addiction without preventing the desired associated feelings. In this connection it is of interest that inhibition of the acetylcholine (ACh) synthetic enzyme, cholinacetyltransferase, by administration of a relatively specific inhibitor of it changed the preference from alcohol to water in mice of the C57/BL strain, which naturally prefer alcohol (Ho et al. 1976; Kissin 1974).

It would be worthwhile to test agents for their antiaddictive properties which, when given with alcohol singly and in combination, would block the synthesis of ACh and its postsynaptic actions in the CNS. If successful in several animal paradigms, some of these agents could be tested in human volunteers. Even if the results should be negative and the hypothesis proven to be incorrect or too limited, the goal of adding harmless antiaddictive but not antieuphorigenic agents to alcoholic drinks to prevent addiction from taking place seems to be a worthwhile one, and other approaches to devise such agents should be sought.

Once addiction has occurred, the best way to begin to extinguish the conditioned responses involved might be to make the intake of alcohol instantly and continuously nonrewarding. This might be achieved by using the other arm of the proposed dyad, blocking some of the pertinent central effects of the catecholamines while leaving the cholinergic ones intact. Many agents are available that can block both the synthesis and the postsynaptic effects of norepinephrine and dopamine. Experiments could be conducted with such agents *included in the alcohol* given to animals in properly designed instrumental conditioning paradigms. The idea here is that extinction would depend on the elimination of the

rewarding aspects of alcohol ingestion. A bonus might accrue to this approach if the elimination of overwhelmingly rewarding aspects of alcohol would allow aversive ones to be uncovered. There would be no attempt to alter catecholamine metabolism at any time except during the intake of alcohol. Drugs that interfere with catecholamine metabolism are known to aggravate withdrawal symptoms (Goldstein 1973; Myers 1978). If this approach were to reach human application, great care would have to be exercised that all alcohol drunk, which at the outset would be available ad libitum, would contain the proper doses of suitable antieuphorigenic blocking agents. A key element in this approach would be to avoid at all costs the ingestion of alcohol not containing the agents, since the addiction might be instantly reinstituted. Because alcohol intake in an established alcoholic to a considerable extent is continued to prevent withdrawal symptoms rather than to achieve pleasure, gradual diminution in intake would be instituted while the individual would be supported with benzodiazepines, valproic acid, lithium, or other nonaddictive agents that may relieve the withdrawal symptoms (Myers 1978). Throughout the treatment, social approval by meaningful individuals in the environment would be given constantly for all evidences of progress. An essential part of the scheme is to prevent withdrawal symptoms. It is desirable for the organism to learn three things: (a) alcohol intake is not rewarding; (b) alcohol intake may be aversive; (c) being without alcohol not only is not aversive, but also can be socially rewarding.

2. Perhaps the earliest neural effects of an acute single dose of alcohol are exerted directly on membranes, and all that follows is a consequence of these effects. When naive neuronal membranes at first are faced with alcohol, their fluidity is increased (Johnson, Friedman, Cooke, and Lee 1980; Johnson, Lee, Cooke, and Loh 1980) and metabolic signals are generated that, if continued or repeated, eventually could lead to compensatory changes in membrane composition, such as increases in the degree of saturation of the fatty acids in membrane lipids, changes in ratios of phospholipid classes, and/or alterations in other membrane constituents. Work concurrently going on in a number of laboratories should give pertinent data in the near future. Two of the immediately important physiological consequences of the membrane fluidization probably are decreases in release of GABA from terminals of inhibitory GABAergic neurons, noted previous-

ly, and the simultaneous specific potentiation of GABAergic postsynaptic action (Davidoff 1973; Nestoros 1980). Together with normal operational levels of GABA, alcohol would give a greater than normal surge of inhibition at the postsynaptic recipient sites of synaptic GABA release at the first instant of contact. One of its effects may be enhancement of binding capacity for GABA at its receptor site (Ticku and Burch 1980). This would give a brief signal for adjustments to take place, which, in the continued or repeated presence of alcohol, eventually could lead to decreases in GABA production (Leitch et al. 1977; Rawat 1974) and in numbers of GABA receptor sites (Ticku and Burch 1980). Signals to major excitatory neurons (Freed and Michaelis 1977), possibly glutamatermic and aspartergic, eventually would lead to hypertrophy of their functions to redress the threatened ascendancy of inhibition. The latter tendency also could be reinforced in many excitatory neurons by inhibitory feedback signals arising from bursting brainstem circuitry. There also could be increases in β-endorphin receptors on GABA neurons and enhancement of the efficacy of the endorphin-releasing neurons that ordinarily inhibit GABA neurons. Exogenously derived alcohol now will have entered on the inhibitory side of equation that determines the balance between inhibitory and excitatory events. In summary, the above formulation postulates compensatory decreases of potency of the GABAergic and increases in the β-endorphin systems and the major excitatory systems with the continued use of alcohol.

The unstable part of the neural equation becomes the inhibitory one because of the difficulty experienced by the alcoholic individual in maintaining a constant level. Although he becomes a virtual alcoholostat, the vicissitudes of everyday life and the necessity for sleep force relatively wide fluctuations to occur in alcohol content of blood and tissues. With continued use of alcohol and the accompanying above-hypothesized compensatory functional atrophic and hypertrophic changes, a drop in blood alcohol levels would create an imbalance in favor of excitatory influences. To this could be attributed many of the discomforts of withdrawal and the concomitant behavioral manifestations—irascibility, restlessness, failure to concentrate, and eventually even seizures. Because the experience of withdrawal is so aversive, there is a tendency to drink more than enough to correct the neural imbalance that gives rise to

it. This leads to further compensatory changes in the directions noted above, so that more alcohol is progressively required to balance out successive losses in inhibitory and gains in excitatory neural potencies. It also leads to periods of behavioral and affective depression, because at higher concentrations than those that are just active on synaptic membranes, such as may occur in overdrinking, alcohol produces decreases in conductile properties of all neurons through its anesthetic actions.

Any cure of alcoholism that requires total abstinence from alcohol or decrease in its intake would be expected to be accompanied by withdrawal symptoms. When the alcoholic part is removed from the inhibitory side of the neural equation, the hypertrophied major excitatory elements (glutamatergic and possibly aspartergic) overbalance the atrophied inhibitory systems (largely GABAergic) and can result in incoordinative overexcitation in every region of the CNS. The imbalances that occur probably are responsible for the neural, behavioral, and psychological manifestations of withdrawal. During alcoholic tolerance and dependence, at least in experimentally addicted animals, there are increases in glutamate functionality (Freed and Michaelis 1978) as well as decreases in the rates of formation and contents of GABA (Leitch et al. 1977; Rawat 1974) and decreases in numbers of postsynaptic GABA receptors (Ticku and Burch 1980). Amino-oxyacetic acid, a GABA aminotransferase inhibitor, ameliorated withdrawal symptoms in alcohol-dependent mice, and picrotoxin, a specific blocker of the postsynaptic action of GABA, made them worse (Goldstein 1973). When alcohol intake is terminated or reduced, compensatory adjustments would be expected to take place with time that would be opposite to those presumed to take place during the development of tolerance and dependence—like playing a motion picture backwards. During this period the nervous system would be in delicate balance at all times with a tendency to local and general seizures.

The ideal conditions for this reversal to take place might be ones in which the compensatory adjustments could occur under physiological conditions without introducing exogenous chemical agents that directly offset the function of one or the other of the suspected major transmitter systems, glutamatergic and GABAergic. Presumably activity of the former might possibly be attenuated by blocking glutamate activity with glutamate diethyl ester (Freed and Michaelis 1978) or proline (Cherkin et al. 1976; Felix and Kunzle 1976; Van Harreveld 1980; Van Harreveld and Strumwasser 1980), and that of the latter might be enhanced by agents that increase postsynap-

tic efficacy of GABA, such as the benzodiazepines (Iversen 1980). The danger of using exogenous agents with some specificity for the affected systems is that the plastic processes required for compensatory readjustment might be incoordinated and addiction might take place to one of the agents employed, as in the case of alcohol. In addition, in the chaotic and variable conditions that would be expected to exist in the CNS during withdrawal, what might work at one time may not be effective at another. It is for this reason that the observation that withdrawal reactions in addicted mice were attenuated by lidocaine is of particular interest (Freund 1973). Indeed, the possibility of the judicious use of local anesthetics in preventing withdrawal symptoms is a particularly attractive one, since the action of these substances probably is not on one or another of the major transmitter systems involved.

In the study of withdrawal symptoms, as with any pathologic phenomenon, one aim is to identify the rate-limiting steps, bottlenecks, so to speak, at which the tools at hand may enable one to choke off the spread of the manifestations of the pathologic state. Often the best strategy is to try to focus on key events as close to the origin of the problem as possible because the consequential, ever-widening ripples at every point of advance of the pathologic process create subsidiary problems that often are unpredictable and may eventually require additional therapies far removed from the original problem. There are multiple causations and many overt manifestations of epileptiform phenomena, such as I suggest withdrawal symptoms to be. What do all of them have in common? The most apparent common denominator and the hallmark of seizures is that principal neurons (P neurons, figure 3), which normally are involved largely as individuals or in small groups in highly specific aspects of information processing in a given neural sector, first begin to fire abnormally frequently when engaged in performing their regular assignments and then join other neurons in the same sector in a series of relatively simultaneous impulses at high frequency in a manner irrelevant to their role in information processing (figure 5). Eventually this may lead to self-sustaining discharges in adjacent and even distant neural sectors, with various behavioral consequences occurring.

The most salient feature that emerges is that in seizures some individual or groups of neurons are firing with maladaptively high frequencies. The firing mechanisms probably are similar in most neurons that show an action potential. It would appear that if a substance would be administered or a procedure performed that

could serve as a low-pass frequency filter with a cutoff point that would still allow neurons to perform their normal functions at the lower frequencies of firing ordinarily required, one might affect events close to the source of origin of seizures. Furthermore, it is possible that such an approach may also give differential effects on through-put principal neurons and on inhibitory interneurons. At least some of the latter may not use action potentials, but may release transmitter as a continuous function of membrane potentials that fluctuate within the release portion of their input-output curves and might maintain transmitter release indefinitely (Graubard 1978). These nonspiking interneurons might be much more resistant to procedures with a relatively specific effect on the spike-generating mechanism. The finding that a number of local anesthetics possess anticonvulsant properties at low levels but are convulsants at higher concentrations suggests that these substances may exert such differential effects. Nature's own frequency filters probably reside at inhibitory synapses. Regionally liberated cybernenes and possibly some circulating factors also probably affect the functions of inhibitory synapses.

Thus, perhaps the most general approach that might be applicable to the treatment and/or prevention of epileptiform discharges in the nervous system would be to affect the conductile properties of nerves that generate action potentials in such a way as to set a ceiling on the frequencies with which they could fire, while having little or no effect on inhibitory interneurons, many of which may not have a spike-generating mechanism. It has been known for some time that local anesthetics exert their action by producing a conduction block in peripheral nerves by blocking transmembrane sodium current (Courtney 1975). In addition, quite aside from knowledge of their mechanisms of action, local anesthetics have been used to prevent or abort a variety of seizures in animals and in humans, suppressing spike generation in cortical epileptic foci and even afterdischarges in electrically stimulated isolated brain slabs (Bernhard and Bohm 1965). It has been found that the nerve-blocking action of local anesthetics is frequency selective (Courtney et al. 1978).

The differential blockade by local anesthetics of sensory impulses in preference to motor impulses possibly may be attributable to the fact that neurons bringing peripheral signals into the CNS (e.g., pain signals) may often fire with higher frequencies than those dealing with motor functions. Frequency-dependent conduction

blocks may play an important role in the analgesia associated with the actions of local anesthetics.

It is particularly important for our present discussion that there now has been a great deal of experience in the use of local anesthetics in the treatment of cardiac arrhythmias (deJong 1977) and in seizure disorders (Bernhard and Bohm 1965). Dosage schedules and precautions in their use in human clinical medicine are well known. There appears to be no reason why they should not be tested for their efficacy in the treatment of alcoholic withdrawal symptoms.

Optimistically, we hope that those working in the field of alcoholism will have devised effective therapeutic approaches in time to salvage the life and health of our hypothetical subject. If not, a bleak downhill fate is in store for him. Continued deterioration of his social relations and his physical health will interact to make him more susceptible to a variety of degenerative diseases of various tissues, including the CNS. Although one may attempt to stem the tide by treating each one of them as the symptoms begin to surface, a walk through the skid row of any large city will quickly convince one that this approach is no more effective than putting a finger in a hole in a dike.

To my knowledge, there currently are no truly effective treatments that predictably can achieve the cure of alcoholism. From the discussion in the preceding sections, let me propose an approach, all aspects of which first should be tested in suitable animal studies. Let us place our hypothetical alcoholic in a friendly and firm, but not austere, environment. Good food and supportive personnel would be available, and so would alcohol ad libitum in the preferred form. All of the latter, however, would contain a previously developed and tested substance that specifically would prevent the euphorigenic effects of alcohol without affecting its taste or anesthetic actions. In addition, at the outset an intravenous infustion of a local anesthetic, e.g., lidocaine, would be begun at the levels in the range of those used to correct cardiac arrhythmias, and careful clinical observations made and subjective reactions noted. The voluntary intake of alcohol would be observed. If the plot of intake over time would reveal a significant decremental trend, then with the assent of the patient, the lowest previous level for a 24-hour period could become the maximal level made available subsequently. The effects of the local anesthetic could be supported by small amounts of a suitable benzodiazepine, if discomfort becomes evident. The latter would be continued only

briefly. If definitive evidence of decreased desire for alcohol should develop, the intravenous administration of the local anesthetic could be substituted by intramuscular administration at intervals. Eventually, when they become available, orally effective preparations of local anesthetics contained in time-release capsules could be given. Finally, all treatment would be stopped when voluntary intake of alcohol is zero. The idea behind this proposed treatment paradigm is to fulfill the criteria previously set forth for the elimination of alcohol under conditions in which there would be a simultaneous approach to those aspects of the condition that are associated with both the "psychological" and "physical" aspects of the addiction, which, of course, are inseparable.

Because of its effects on the membranes of all cells that it contacts, potentially alcohol can produce structural and metabolic incoordinations at cellular and tissue levels everywhere in the body. To some extent, susceptibility to its toxic effects must be genetically determined, but factors of age, presence of viral and bacterial agents, and nutritional status also must play important roles. Effects on the immune system may increase susceptibility to infection and compound the initial damage that alcohol causes by facilitating autoimmune reactions. However, although extremely important in the consideration of the problem of an individual patient, the thousands of published reports on the above and similar subjects have not been useful in devising generally useful approaches in the prevention and/or treatment of alcoholism.

Summary

The approach taken has been broad and frankly speculative. Schemata are presented for considering normal and maladaptive behavior of an organism in its environment, for the operation of various major regions and circuits of the nervous system in such behavior, and for the basic structures of the units and modules of the components of the nervous system. It is suggested that in behavioral sequences, innate or learned, preprogramed circuits are released to function at varying rates and in various combinations largely by disinhibition of pacemaker neurons whose activities are under control of tonically active command neurons, many of which use GABA as a transmitter. The manifestations of many diseases of the nervous system and of nonneural tissues as well are considered

to be consequences of incoordinations between inhibitory and excitatory processes in the CNS, of which local or global seizure-like activity is considered to be prototypic.

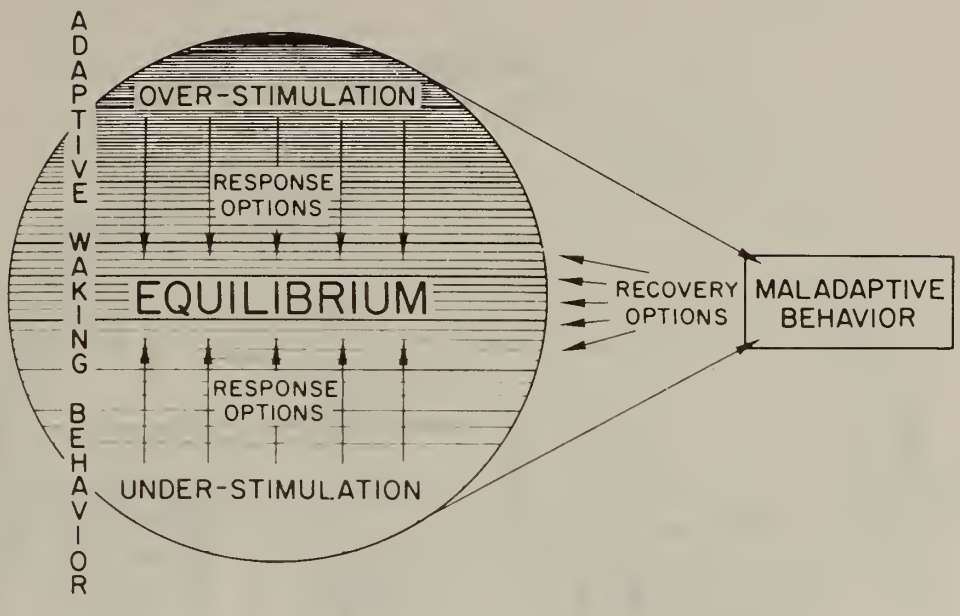
The addictive effects of alcohol are postulated to be consequential to its fluidizing effects on neural membranes after rapid entry into tissues. There may follow decreases in releases of a variety of neurotransmitters from nerve terminals, the key one being GABA, and facilitation of electrotonic communication between neurons via gap junctions. The latter conditions may then lead to oscillatory discharges of euphorigenic (catecholamine-releasing) and memory-enhancing (cholinergic) circuits in the brainstem, whose activities ordinarily are coupled to anticipation of solutions to problems related to consummatory activities. The discharge of these circuits in the presence of alcohol becomes rapidly and firmly associated through conditioning with the presence of alcohol itself and with the environment in which it is taken. It is suggested that it may be possible to develop nontoxic, palatable agents that, when added to alcoholic beverages, may allow the rewarding aspects of drinking (catecholaminergic) to remain while blocking the memorial concomitants (cholinergic). According to this formulation, such agents should prevent addiction or make it less likely, while allowing alcohol to be drunk freely.

A rationale is presented for the development of compensatory atrophic decrements in indigenous inhibitory neural elements and hypertrophic increases in excitatory elements and for the emergence of alcohol as an essential exogenous inhibitory influence. It is suggested that the alcoholic eventually becomes an alcoholostat, one of whose chief goals is to maintain levels of alcohol in blood and tissues that are sufficient to prevent withdrawal symptoms. The latter result from seizure-like local and global states in the CNS that occur when coordination between excitation and inhibition is disturbed, with excitation predominating. Because of the imperfections of the detecting systems and vicissitudes in alcohol intake, wide variations in blood levels may occur, with consequent swings between neural and behavioral depression and overexcitation. Use of local anesthetics, which act essentially as low-pass frequency neural filters, in preventing the symptoms of withdrawal is recommended. This is preferable to the administration of substances like barbiturates, bromide, and so on, which are addictive in their own right.

The above considerations led to the suggestion of the devisal of a therapeutic technique that would make it nonrewarding to drink

and nonaversive not to drink for a period of time. During that period of time, compensatory changes could take place in the neural machinery to restore the normal balance between indigenous inhibitory and excitatory neural systems to a level that is functionally effective and affectively tolerable.

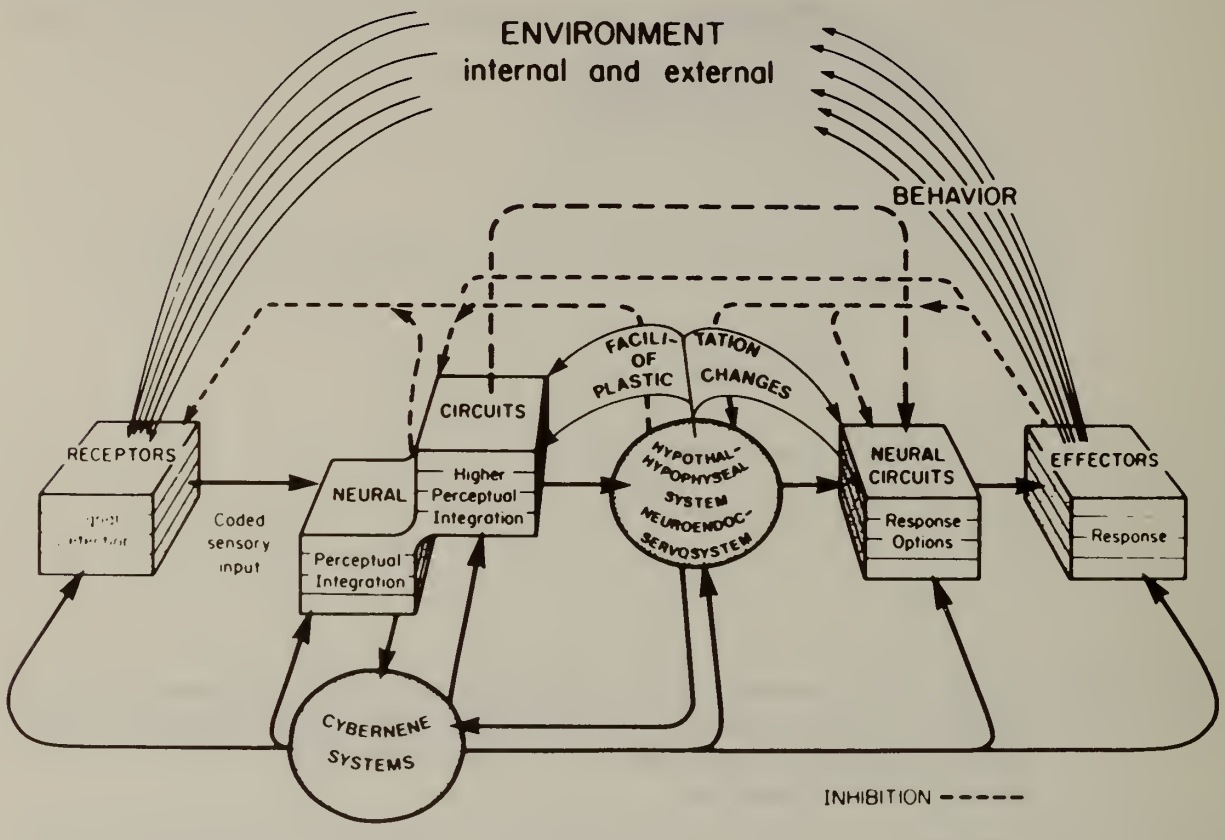
Figure 1. A "Homing" Model for an Organism in Its Environment



SOURCE: Crook and Gershon, eds., *Strategies for the Development of and Effective Treatment for Senile Dementia.* New Canaan, Conn.: Mark Powley Associates, Inc., 1981. Copyright 1981 by Mark Powley Associates, Inc.

Note: Environment = Internal environment plus internal representation (abstraction) of external environment.

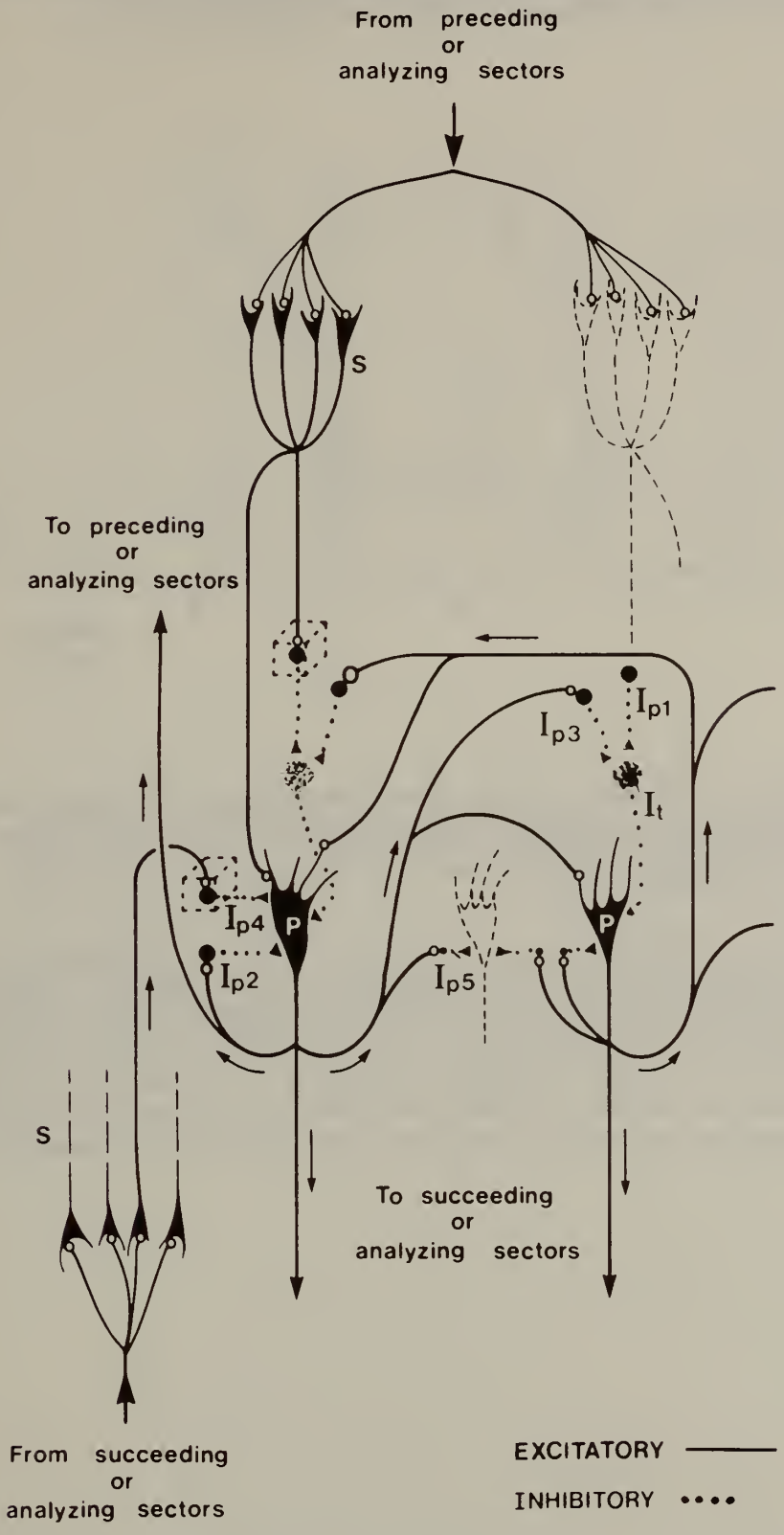
Figure 2. A Model for Behavior Showing the Major Systems Involved in Informational Transactions



SOURCE: Crook and Gershon, eds., *Strategies for the Development of and Effective Treatment for Senile Dementia.* New Canaan, Conn.: Mark Powley Associates, Inc., 1981. Copyright 1981 by Mark Powley Associates, Inc.

Note: The intimate relationships of the through-put (dark) and cybernene (light) neural systems are indicated.

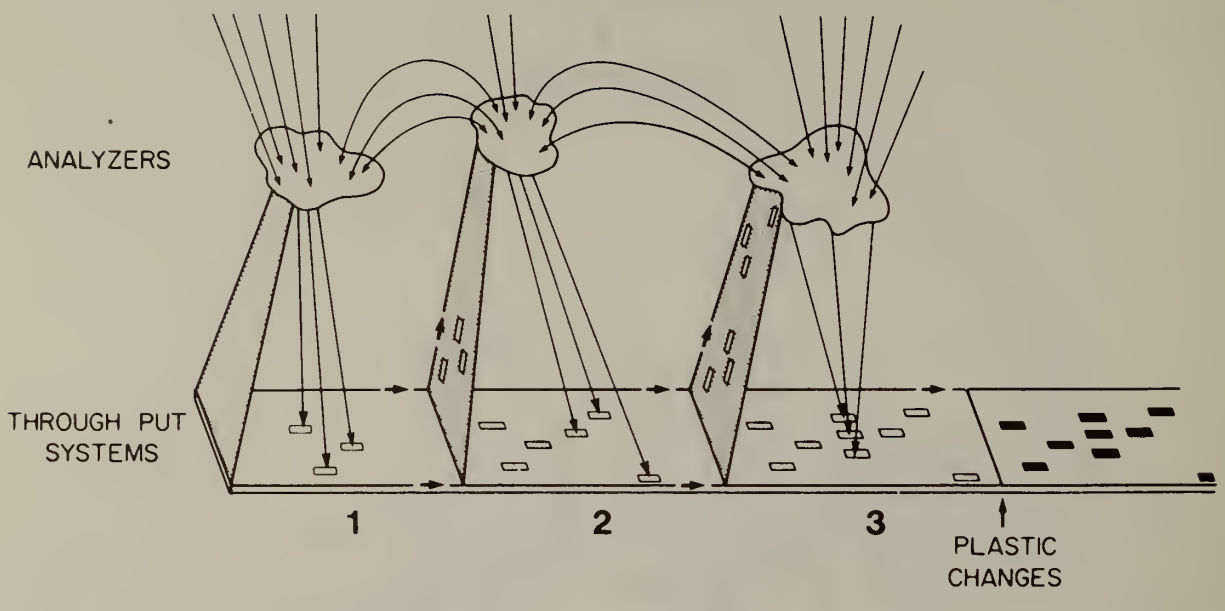
Figure 3. Outline of "Minimal" Basic Neural Circuitry



SOURCE: Crook and Gershon, eds., *Strategies for the Development of and Effective Treatment for Senile Dementia.* New Canaan, Conn.: Mark Powley Associates, Inc., 1981. Copyright 1981 by Mark Powley Associates, Inc.

Note: The details of this model are discussed in the text. P, principal or pacemaker neurons; I_t, tonically active inhibitory neurons; I_p, phasically active inhibitory neurons; S, excitatory satellite neurons.

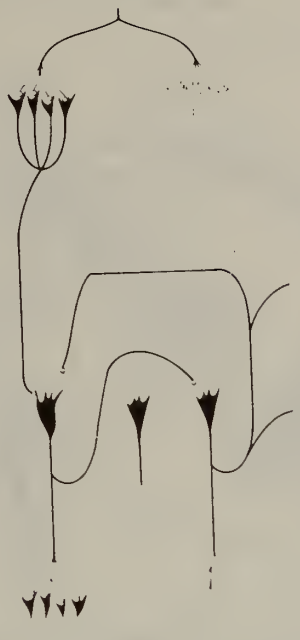
Figure 4. A Model for Relationships Between Through-Put System and Analyzing Sectors



SOURCE: Crook and Gershon, eds., *Strategies for the Development of and Effective Treatment for Senile Dementia.* New Canaan, Conn.: Mark Powley Associates, Inc., 1981. Copyright 1981 by Mark Powley Associates, Inc.

Note: Minimally, a bifurcation of the flow of neural information from a particular sector takes place into direct through-put channels and also into those leading to analyzing regions such as the cerebellum, hippocampus, basal ganglia, and association cortex. The arrow indicates that plastic changes (increases of connectivities) occur at some synapses when it is signaled that an appropriately adaptive response to a new situation has been achieved by the organism.

Figure 5. Representation of a Module or Basic Neural Circuit Without Inhibition



SOURCE: Crook and Gershon, eds., *Strategies for the Development of and Effective Treatment for Senile Dementia.* New Canaan, Conn.: Mark Powley Associates, Inc., 1981. Copyright 1981 by Mark Powley Associates, Inc.

Note: The inhibitory neurons may become ineffective because of failure to release inhibitory neurotransmitter, becasue of postsynaptic ineffectiveness of released transmitter, or for both reasons. This is meant to depict active neural circuitry during a paroxysmal discharge.

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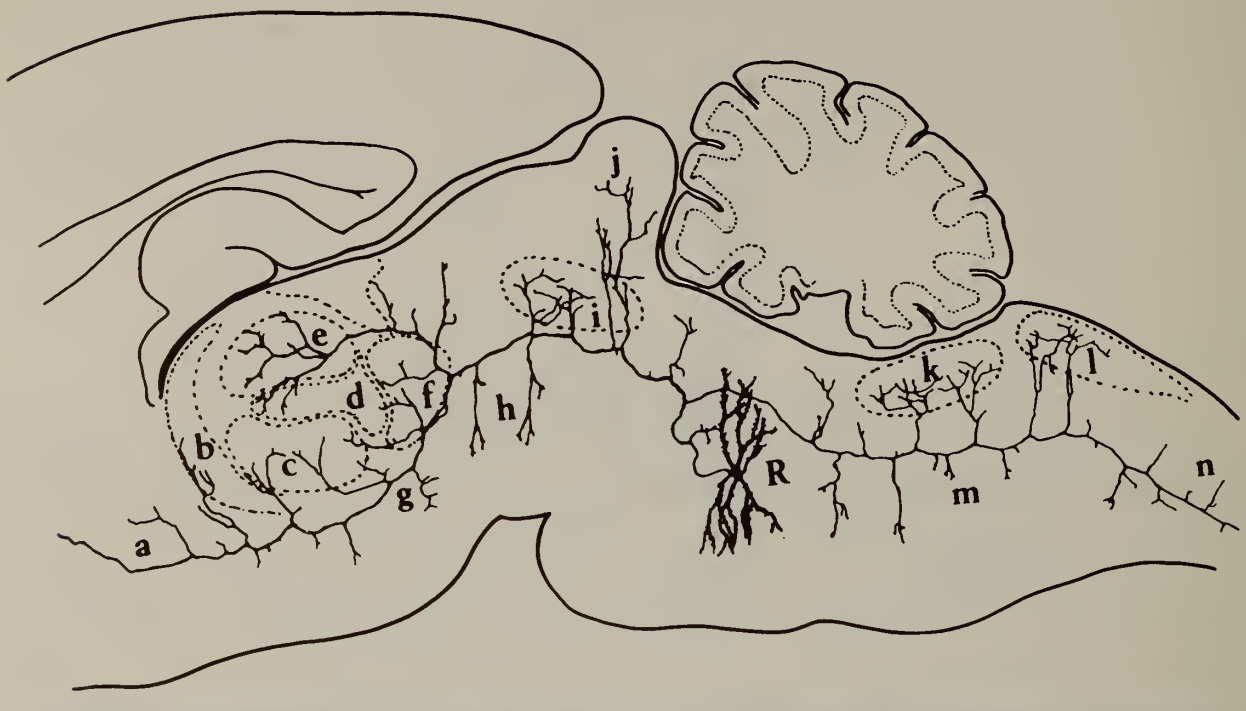
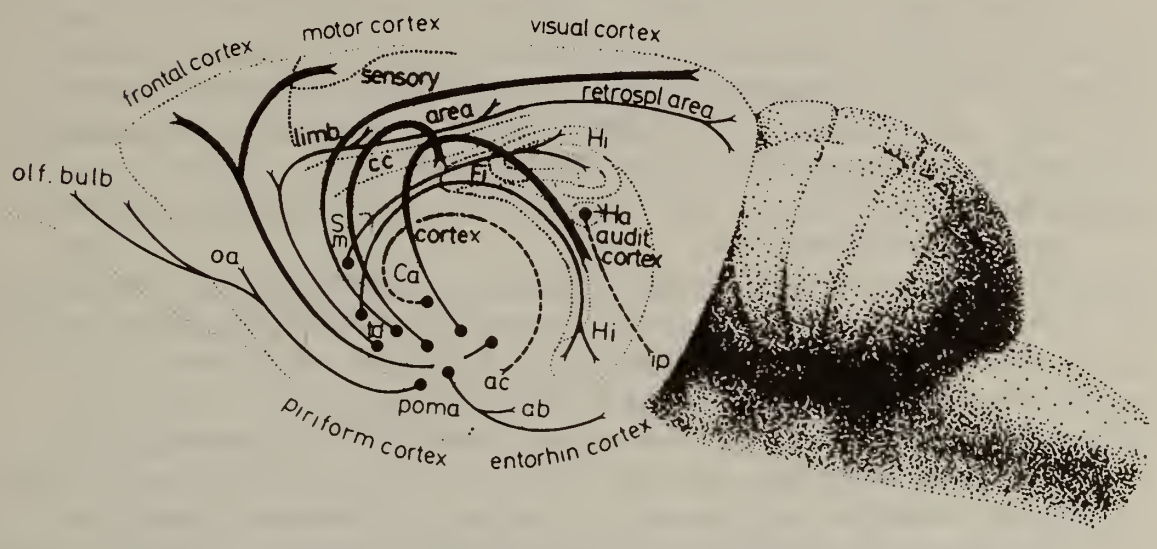
Figure 6. Sagittal Section Showing Ascending and Decending Axon System of Single Reticular Neuron in the Nucleus Gigantocellularis



SOURCE: Crook and Gershon, eds., *Strategies for the Development of and Effective Treatment for Senile Dementia.* New Canaan, Conn.: Mark Powley Associates, Inc., 1981. Copyright 1981 by Mark Powley Associates, Inc.

Note: Note ascending and descending branches and rich collateral fibers entering the following areas: a, basal forebrain; b, nucleus reticularis thalami; c,d,e, complex of medial and intralaminar thalamic nuclei; f, centromedian-parafascicular complex; g, zona incerta; h, mesencephalic tegmentum; i, oculomotor-trochlear nerve nuclear complex; j, inferior complex; k, hypoglossal nucleus; 1, nucleus gracilis; m, medullary reticular formation; n, spinal cord. Golgi modification, young rat. Originally published in Brazier, M.A.B. *The Electrical Activity of the Nervous System.* 3d ed. London: Pitman, 1968; Baltimore: Williams and Wilkins 1968).

Figure 7. Schematic Drawing Summarizing the Direct Monosynaptic Cholinergic Pathways From Magnocellular Nuclei of the Basal Forebrain of the Rat to the Cortex



SOURCE: Wenk et al. 1980; reprinted with permission of the authors.

Note: Neocortical fields receive their cholinergic innervation from scattered cell groups situated in the substantia innominata (dark neurons not designated lying outside and between the nuclei of origin innervating paleocortical and archicortical fields). Olfactory bulb and entorhinal cortex are innervated by cholinergic neurons of the nuc. preopticus magnocellularis (poma); the hippocampal formation and the limbic cortex from cholinergic cells of the medial septum (sm) and Broca's diagonal tract nucleus (td). Abbreviations: ab, nuc. amygdaloideus basalis; ac., nuc. amygdaloideus centralis; Ca, commissura anterior; CC, corpus callosum; Ha, nuc. habenulae lateralis; Hi, hippocampus; ip, nuc. interpeduncularis; oa, nuc. olfactorius anterior; Fi, fimbria hippocampi; Sm, stria medullaris thalami; td, nuc. tractus diagonalis (Broca).

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Biological Sensitivity to Alcohol

Arthur R. Zeiner

Abstract

A variety of substances (disulfiram, calcium carbimide, chlorpropamide, among others) lead to an adverse reaction to alcohol. These substances interfere with the acetaldehyde metabolizing step so that acetaldehyde builds up after an acute dose of ethanol. Acetaldehyde has been related to adverse autonomic reactions that make ethanol ingestion unpleasant. The adverse reaction to ethanol after pretreatment with disulfiram forms the basis of aversion therapy with alcoholics. In some people this adverse reaction to alcohol occurs without pretreatment with disulfiram. Some evidence suggests that acetaldehyde also builds up in those people having the alcohol sensitivity after an acute dose of alcohol. Another line of evidence suggests that racial and individual differences in atypical alcohol and acetaldehyde dehydrogenases may serve as the physiological basis of biological sensitivity to alcohol. Those people who possess the biological sensitivity to alcohol are at low risk for becoming alcoholics or alcohol abusers because the unpleasant effects of drinking alcohol far outweigh its pleasant euphoric effects; however, those people who do not have sensitivity may be at risk for becoming alcoholics if they turn to alcohol when under stress. It is our hypothesis that racial and ethnic differences in alcohol use and abuse may be accounted for, in part, by racial and ethnic differences in biological sensitivity to alcohol. The biological sensitivity, in turn, is based on atypical alcohol and more particularly acetaldehyde dehydrogenase enzymes. If these hypotheses are true, then it may be possible to develop physiological tests that could assay for and indicate risk for alcoholism. This paper reviews the evidence for these related hypotheses and presents our most recent findings on the topic.

Recent evidence suggests that there may be in part a biological basis for alcohol aversion and/or biological sensitivity to alcohol. This paper is a review of the evidence. The review focuses on (a) studies that demonstrate a naturally occurring biological sensitivity to alcohol similar to that seen with the disulfiram-alcohol reaction; (b) a differential distribution of alcohol and acetaldehyde

dehydrogenase liver enzymes among racial groups; and (c) gender differences in ethanol pharmacokinetics and ethanol intake related to female sex steroid concentrations.

Biological Sensitivity to Alcohol

Aversion therapy for alcoholism is based on the early work of Hald, Asmussen, and Jacobsen. These investigators demonstrated in the late 1940s that pretreatment with disulfiram resulted in aversive consequences if alcohol was ingested (Asmussen, Hald, Jacobsen, and Jorgenson 1948; Asmussen, Hald, and Larsen 1948; Hald and Jacobsen 1948). Hald and Jacobsen (1948) demonstrated that breath acetaldehyde concentration is increased sevenfold to ethanol intake after pretreatment with disulfiram. Disulfiram blocks the acetaldehyde metabolizing step, and concentrations of the substance build up in the body as a result.

Recent research suggests that elevated acetaldehyde concentrations may be seen in people who have an alcohol sensitivity without pretreatment with disulfiram. The sensitivity is not all or none but seems to be graded. It also has a differential racial distribution such that a greater percentage of the Oriental population possesses the sensitivity than do either Caucasian or black races. Zeiner (1980) reviewed the literature relating to the disulfiram-alcohol reaction and biological sensitivity to alcohol. In both cases the physiological reactions are characterized by (a) facial flushing, (b) increases in heart rate, (c) decreases in blood pressure, (d) increases in cardiac output, (e) increases in rate and depth of respiration, (f) increases in peripheral vasodilation as indexed by pulse wave amplitude changes, temperature changes, or blood flow changes, and (g) increases in both skin conductance level and frequency of nonspecific skin conductance responses. The size of the observed physiological changes correlated with either blood or breath acetaldehyde concentrations.

Alcohol Sensitivity Among Orientals

Ijiri (1974) and Mizoi et al. (1979, 1980) performed a series of experiments in which they investigated cardiovascular reactions, urinary catecholamine excretion, ethanol pharmacokinetics, and acetaldehyde concentrations to an acute dose of ethanol among

groups of Japanese showing a flush response and a group not showing a flush response. Groups did not differ from each other on a baseline prior to alcohol ingestion on cardiovascular measures, endogenous acetaldehyde concentration, or urinary catecholamine excretion. Nor did the groups differ reliably from each other on ethanol pharmacokinetics, e.g., time to peak blood alcohol concentration (BAC), peak BAC attained, or ethanol clearance rate. However, the group showing a facial flush showed remarkable postethanol increases in acetaldehyde concentration, urinary catecholamine release, heart rate increase, and facial temperature. Such changes were minimal or not evident in the group not demonstrating a facial flush. The authors concluded that acetaldehyde concentrations were related to the observed effects.

Earlier, Wolff (1972, 1973) had assayed for dysphoric symptoms and autonomic involvement to a small dose of ethanol among Oriental and Caucasian groups. A much larger percentage of the Orientals than of the Caucasians demonstrated facial flushing and dysphoric symptoms. Ewing et al. (1974), Hanna (1977), and Sanders et al. (1980) replicated and expanded on the Wolff findings.

Two experiments, following the lead of Ijiri (1974), investigated acetaldehyde concentrations for the same acute dose of alcohol among different racial groups. Reed et al. (1976) demonstrated that American Indians obtained the highest acetaldehyde concentrations for a given dose of alcohol. Orientals were intermediate (they also received a lower dose), and Caucasians had the lowest acetaldehyde concentration for the given dose of alcohol. Zeiner et al. (1979) compared male Chinese exchange students with Caucasian students from the University of Oklahoma. For the same dose of alcohol (0.3 cc/lb 190 proof alcohol in a 1:4 orange drink mix), the Oriental students reached an acetaldehyde concentration twice as high as that reached by Caucasians. A much larger percentage of the Orientals showed a facial flush than did the Caucasians (replicating earlier work by Wolff, Ewing, and Hanna). Cardiovascular changes (heart rate increases) were directly related to observed acetaldehyde concentration attained (replicating findings by Sauter et al. 1977). Results of these experiments are consonant with the view that acetaldehyde buildup may mediate the dysphoric symptoms and alcohol intolerance.

Facial Flushing Among Other Groups

Although facial flushing, dysphoric symptoms to alcohol, cardiovascular changes, and increased acetaldehyde concentrations are most clearly delineated in Oriental populations, such findings have also been observed in other groups. For example, Wolff (1973) demonstrated facial flushing to a small dose of alcohol among American Indians that did not differ from the flush observed among Orientals. Facial flushing, however, is but one facet of the response, and other indexes, such as acetaldehyde concentration, were not measured by Wolff. Zeiner at al. (1976) observed facial flushing to an acute dose of alcohol among Tarahumara Indians in Mexico. In a followup study, Zeiner et al. (1977) demonstrated that although Tarahumara Indians showed a facial flush to an acute dose of alcohol, either other autonomic measures (facial temperature, skin potential responses) did not discriminate between Tarahumara Indians and Caucasians or the Tarahumara were actually less reactive to alcohol (heart rate change) than were the Caucasians. Unfortunately, neither of these experiments measured acetaldehyde concentrations. The experiment by Reed et al. (1976) observed higher acetaldehyde concentrations among Indians than among either Orientals or Caucasians, but their data for the Indian group may have been atypical. Zeiner et al. (1979) demonstrated that about 10 percent of Caucasians also show a facial flush to an acute dose of alcohol.

Atypical Alcohol and Acetaldehyde Liver Enzymes

An area of research that may be related to biological sensitivity to alcohol deals with atypical alcohol and acetaldehyde dehydrogenase liver enzymes. Von Wartburg (1977) and his colleagues (1965, 1968) embarked on a series of investigations to characterize the distribution of these atypical liver enzymes among different populations. Of interest was the possibility that possession of these enzymes might lead to more rapid ethanol metabolism and/or greater buildup of acetaldehyde. Greater acetaldehyde production, in turn, might mediate dysphoric effects observed with ethanol ingestion.

Von Wartburg and Schurch (1968) showed that about 4 percent of the Swiss population had these atypical enzymes. Edwards and Evans (1967) came up with a figure of 4 percent of British subjects having the atypical alcohol dehydrogenase enzymes. Conversely, among Japanese, studies indicate that from 85 to 98 percent of the people may possess the atypical alcohol dehydrogenase enzyme forms (Fukui and Wakasugi 1972; Ogata and Mizohata 1973; Stamatoyannopoulos et al. 1975). Harada et al. (1980) replicated and extended on the earlier findings with Japanese liver specimens. They found that 85 percent of liver specimens from autopsied Japanese contained the atypical ADH enzyme. Further, they demonstrated two major isoenzyme bands for acetaldehyde dehydrogenase (ALDH) in these specimens, one type having a faster migrating band (low Km for acetaldehyde) and a second type having a slower migrating isoenzyme (high Km for acetaldehyde). Fifty-two percent of these samples possessed only the slower migrating isoenzyme. Harada et al. (1980) also found that the usual phenotype was inhibited 10 to 30 percent by disulfiram, whereas the unusual phenotype was inhibited up to 90 percent. They concluded that persons possessing the unusual phenotype may experience delayed oxidation of acetaldehyde after an acute dose of ethanol rather than the production of more or greater amounts of acetaldehyde from faster alcohol metabolism.

Teng et al. (1979) extended this line of research by investigating the incidence of atypical alcohol dehydrogenase enzymes among Chinese and Indian populations from West Malaysia. They discovered that 89 percent of the Chinese but none of the Indians carried the atypical ADH form.

The findings reviewed above are consonant with the hypothesis that altered alcohol metabolism, or perhaps more accurately, the buildup of acetaldehyde by either faster ethanol metabolism or faulty acetaldehyde metabolism, may be the basis of dysphoric symptoms after an acute dose of ethanol and biological sensitivity to alcohol. Studies are needed that combine the atypical ADH and ALDH literature with ethanol and acetaldehyde pharmacokinetic studies and autonomic concomitants to an acute dose of alcohol among different racial groups having a differential distribution of these enzymes.

Sex Steroid Alcohol Interactions

Several animal studies have demonstrated that rats will decrease their voluntary intake of ethanol after receiving estrogen injections (Aschkenasy-Lelu 1958, 1960*a*,*b*). Further, during the estrus stage, at a time of elevated estrogen concentrations, rats will drink less alcohol than at other times (Aschkenasy-Lelu 1960*a*,*b*). In related experiments, Mardones (1960) noted that diethylstilbesterol significantly reduced ethanol intake in either normal or gonadectomized males and females, whereas no such effects were demonstrable with testosterone. Emerson et al. (1952) found that high doses of estradiol benzoate reduced ethanol consumption in deer mice, whereas progesterone administered in massive doses did not alter ethanol intake in the same species. Ericksson (1969) tested for the effects of ovariectomy and contraceptive sex steroids on voluntary alcohol consumption in 3-month-old female rats. Prior to ovariectomy, experimental and control groups did not differ on ad lib alcohol intake. After ovariectomy, there was a small decrease in ethanol intake among experimental animals. Administration of contraceptive hormones had a strong inhibiting effect on ethanol consumption of both ovariectomized and control rats. Einarsson et al. (1975) demonstrated that estrogenic compounds given in higher than physiological doses have a suppressing effect on microsomal steroid metabolizing activities.

With humans, Little et al. (1976) noted that there was a sharp decrease in the use of alcoholic beverages during pregnancy. This effect did not seem to be due to fears about fetal alcohol syndrome and risks to the unborn fetus; instead, patients cited adverse physiological effects as the major reason for the decline in drinking during pregnancy. In a followup study, Little and Streissguth (1978) demonstrated that during pregnancy female alcoholics also decreased their alcohol intake.

Jones et al. (1975) found that women taking oral contraceptives drank significantly less alcohol than did control females not on oral contraceptives. Zeiner and Farris (1979) extended the generality of these findings by testing American Indians on oral contraceptive steroids and a group of controls not on contraceptive steroids. Groups did not differ on age, education, socioeconomic level, height, or weight. However, the group on oral contraceptive steroids demonstrated significantly lower alcohol intake than did controls.

Evidence that decreased ethanol intake during pregnancy or in females on oral contraceptives might have the same basis as biological sensitivity for alcohol comes from a recent experiment by Kegg and Zeiner (1980). In this experiment, birth control pill effects were tested on ethanol pharmacokinetics, acetaldehyde concentration, and cardiovascular concomitants of 0.52 gm/kg ethanol in a 1:4 orange drink. Two groups of females (seven each) either on oral contraceptive steroids or not on birth control pills were tested twice (once on day 1, the first day of menstrual flow, and once on day 26 of the menstrual cycle). Order of testing was counterbalanced, and groups were equated on height, weight, education, and socioeconomic level. All were at least 4 hours' food-deprived at the time of testing. Blood alcohol concentration and acetaldehyde concentration were determined directly from breath samples via a gas chromatograph every 10 minutes from drinking. Cardiovascular measures were recorded noninvasively before each breath sample via a microprocessor-based recorder. Subjects reached a reliably higher peak blood alcohol concentration on day 1, at a time of low estrogen and progesterone. Acetaldehyde concentration at peak BAC was significantly higher on day 26 than on day 1. Acetaldehyde concentration increased reliably more from day 1 to day 26 in the group on oral contraceptives than in the control group not on oral contraceptives. The oral contraceptive group also had a reliably higher diastolic blood pressure both during baseline and at peak BAC measures than did the control group not on oral contraceptives. These data are consonant with the view that decreased ethanol intake during pregnancy or in females on oral contraceptive steroids may be due to increased acetaldehyde concentrations for a given dose of alcohol over that achieved by females who are not pregnant or not on birth control pills. To our knowledge, definitive answers on these issues are not available but await further research.

In summary, this review proposes that (a) there are individual and racial differences in biological sensitivity to alcohol; (b) the biological sensitivity is graded and is not all or none; (c) there are racial and ethnic differences in atypical alcohol and/or acetaldehyde dehydrogenase liver enzymes; (d) these enzymes may form the basis of biological sensitivity to alcohol; and (e) differences in sensitivity and/or reactivity to an acute dose of ethanol as a function of menstrual cycle, pregnancy, or oral contraceptive steroid intake may also have increased acetaldehyde buildup as

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their common basis. Further research is needed to explore these relationships.

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Influence of Lithium Carbonate on Cellular Protein Synthesis*

Maria E. Rosnowska and Sujata Tewari

Abstract

The authors present the results of their research on long-term effects of lithium treatment on brain and kidney ribosomal protein synthesis in rats. Six-week intragastric intubation of 150 mg/kg/day or 225 mg/kg/day of lithium carbonate resulted in inhibition of kidney protein synthesis at both concentrations and in stimulation of the brain ribosomal system, but only at the lower concentration. Increased lithium concentration resulted in greatly reduced stimulation.

Introduction

Lithium carbonate is regarded as a specific pharmacological agent for the treatment of manic phase and manic-depressive illness and in the treatment of alcoholism. Clinical studies have reported that Li^+ had a direct effect in reducing alcohol consumption by chronic alcoholics (Kline et al. 1974) and is effective in the treatment of alcohol withdrawal symptoms (Sellers et al. 1976). In a recent study, Coppen (1980) reported that Li^+ therapy seemed to be most successful only in those patients who were depressed. Clinically, the onset of Li^+ action has been found to be 7 to 10 days after treatment, with normalization occurring in 1 to 3 weeks. However, following the withdrawal of Li^+, it has also been reported that manic episodes tend to recur. A distinct advantage of Li^+ therapy is that the antimanic effects of Li^+ are exerted without the presence

NOTE: Figures and tables appear at end of paper.

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of sedation (Gerbino et al. 1978). Li^+ is also considered to be a teratogenic agent, as this drug is known to pass freely into the mother's milk. It has been established clinically that mothers requiring lithium carbonate therapy should not breast feed their infants because of high levels of Li^+ in the milk (one-half of the mother's serum lithium level) and in the infant (one-half to one-third of the mother's serum level) (Goldberg and Dimascio 1978). Following administration, lithium is virtually entirely cleared by the kidneys. Thus, it is generally recognized that the renal function is of critical importance in Li^+ therapy, particularly as the margin between the therapeutic and toxic serum concentration is small. Following the administration of a single dose of Li^+, approximately two-thirds of the dose is excreted with 24 hours, with the remainder being slowly excreted over 10 to 15 days (Hollister 1978). Biochemically, Li^+ is known to affect ribonucleic acid (RNA) metabolism in the brain (Dewar and Reading 1974). Furthermore, chronic lithium treatment has been shown to reduce cellular cytoplasmic RNA content and to dissociate E. coli ribosomes at 0.2 M concentration (Huot et al. 1972; Suzuka and Kaji 1968). It has been further observed that the changes in mood in manic-depressives closely followed the changes in daily uric acid excretion, which was increased markedly during the early phase of natural or lithium-induced remission from both hypomanic and depressive episodes. Because uric acid is the major end product in the purine metabolism and brain RNA metabolism is known to be affected by Li^+, it is necessary to determine the effects on the brain and kidney protein synthesis under these conditions.

Very little is known about the long-term effects of Li^+ on the protein synthetic properties of ribosomes. Because of the important role that protein synthesis plays in cerebral function, the present report investigated (a) the long-term effects of Li^+ treatment on the in vitro protein synthesis by the brain and kidney ribosomes, (b) whether Li^+ administration produces similar or dissimilar effects on the protein synthetic activities in these two tissues, (c) the possible interrelationship of the observed effects between these organs, and (d) whether the effects on kidney are primary or secondary effects to those observed in the brain. Data obtained from these studies may be extended to understand both the basic neurochemical reactions underlying the manic-depressive illness and the nephrotoxic effects of Li^+. This report has examined the state of protein synthesis in brain and kidney cell free systems following the lithium carbonate treatment.

Materials and Methods

All chemicals were of reagent A Grade. Adenosine triphosphate (ATP) and guanosine 5′-triphosphate (GTP) were purchased from the Sigma Chemical Company, St. Louis, MO; (^{14}C)leucine (specific activity 340 mc/mmole) was purchased from New England Nuclear Company, Boston, MA; and lithium carbonate capsules were obtained from Philips Roxane Laboratories Inc., Columbus, OH. Two-month-old male Sprague-Dawley rats obtained from the Charles River Company, Boston, MA, were divided into control and lithium-treated groups. The rats in the lithium-treated groups were given 75 mg/kg/day of lithium carbonate in water by intragastric intubation between 8 a.m. and 9 a.m. five times during the first week. In the beginning of the second week, the dosage was increased to 150 mg/kg/day or 225 mg/kg/day. The controls were intubated with an equal volume of water. The treatment was continued for the desired time period, and brains and kidneys were removed for the isolation of ribosomes and the precipitable fraction of pH5 enzymes (Tewari and Noble 1971). The measurement of (^{14}C)leucine incorporation into protein was carried out by procedures already established in this laboratory (Tewari et al. 1980). The protein content of the subcellular fractions was determined by the procedures of Lowry et al. (1951). The RNA content was measured by the warm sodium dedecyl sulfate extraction procedures of Tewari et al. (1980). The urine and plasma levels of lithium were determined by mass atomic spectrophotometry.

Results

Animal Treatment Groups

Table 1 describes the three basic treatment groups and the number of animals used per group. Sixty 2-month-old male Sprague-Dawley rats were divided into three groups: control, Li^+-150, and Li^+-225 groups. Rats were randomly assigned to each group and housed individually. The treatment conditions have already been described in the text. The animals were housed in the vivarium and kept under 12-hour dark and light cycles.

Lithium Effects on Weight Gain, Volume of Water Ingested, and Urinary Output

Under each treatment condition the weight gain, volume of water consumption, and urinary output were routinely monitored and comparisons made with the control group. Table 2 presents data obtained on the weight gain and the volume of water ingested by the rats following the intragastric administration of Li^+. Following lithium administration, a small increase could be observed in the body weights, suggesting that the rats may be retaining some water. Results clearly show that the rats undergoing Li^+ treatment had a marked increase in water consumption in both groups (280 percent for the Li^+-150 group and 322 percent for the Li^+-225 group) when compared to the controls. Also, consistent with the increased water consumption was the pronounced rise in the urinary output, which was 438 percent over controls in the Li^+-150 group and 594 percent in the Li^+-225 group. Effects of lithium were further examined on the weight of different organs such as the brain, kidney, liver, and testes. Essentially, the chronic administration of Li^+ at both concentrations did not have significant effect on the wet weight of these organs (table 3).

Lithium Levels in Urine and Serum

To determine whether these animals could effectively maintain chronic lithium levels in the blood, both urine and serum samples were monitored. Table 4 and figure 1 show the lithium levels in the urine and serum of rats following chronic lithium treatment. As might be expected, higher Li^+ levels were present in 24-hour urinary excretion in the Li^+-225 group when compared to the Li^+-150 group, with the control group exhibiting insignificant values. Similar to the urinary samples, the serum lithium levels were continuously monitored during the entire 6 weeks of experimentation. During the first week when animals received 75 mg/kg of the drug, a small increase in serum Li^+ levels was present at both concentrations when compared to the control values. When the Li^+ dosage was stepped up to 150 mg/kg or 225 mg/kg, a sharp increase in lithium levels was obtained following 1 week of treatment. These concentrations were then maintained at almost the same level during the subsequent weeks of treatment. The control group at all times demonstrated negligible serum Li^+ levels.

Effects of Li^+ on the Incorporation of (^{14}C)leucine Into Nascent Polypeptide Chains

The amino acid incorporation into protein under in vitro conditions was studied by incubating brain and kidney ribosomes and pH5 enzyme fractions at 10, 30, and 60 minutes. Data from these experiments are given in figures 1 and 2. Results show that Li^+ exerts differential effects on the in vitro incorporation of (^{14}C)leucine into protein in these two organs. At all time points, the kidney ribosomes (figure 2) consistently showed significant decreases in the in vitro protein synthetic activity following chronic Li^+ treatment at both the low and the high concentrations. The inhibition was greater with the Li^+-225 group than with the lithium-150 group, and at 60 minutes was 65 percent and 34 percent of the control activity, respectively. These inhibitory effects were present at all time points of incubation. Ribosomes from the brain not only demonstrated a higher ability to incorporate (^{14}C)leucine into protein at all time points but also responded in a different manner to lithium treatment when compared to kidney ribosomes (figure 3). Lithium carbonate was found to be highly stimulative to brain protein synthesis at 150 mg/kg concentration. In contrast to the kidney, where inhibition of protein synthesis was even more pronounced at lithium concentrations of 225 mg/kg, no further increases could be obtained in the cerebral tissue on the incorporation of (^{14}C)leucine into protein. Rather, at this concentration (225 mg/kg), Li^+ considerably lowered the increased level of activity induced by 150 mg/kg dosage, bringing it closer to the control levels. Only a small increase in the in vitro protein synthetic activity of brain ribosomes was obtained in the Li^+-225 group. These effects of lithium were observed in all time points of incubation. Thus, it appears that, at least in the brain, the effects of lithium on protein synthesis may be dose dependent. Experiments are in progress to further examine these issues.

Discussion

In recent years lithium therapy has increasingly been considered in the management of chronic manic conditions and manic-depressive illness. Several laboratories have investigated the specificity of lithium therapy to manic-depressive states. While the neurochemi-

cal mechanisms involved in the manic-depressive illness are as yet unknown, some experimental data are now available showing lithium-induced effects on specific neural enzymes. In this regard, specific enzymatic activities were increased or decreased following lithium treatment (Bera and Chatterjee 1976). In the present study, we show that chronic lithium treatment of male Sprague-Dawley rats for up to 6 weeks results in substantial weight gain, especially at high concentrations of lithium. The weight gain is partly caused by increased water retention, as substantiated by the present observation of increased water intake and large urinary output. The water intake increased from 41 ml/day for the control group to 132 mg in the Li^+-225 group. Similarly, total urinary output was also increased from 16 ml in the control group to 95 ml in the experimental group. Under the present treatment condition, although the body weights were increased, specific weight of brain, kidney, and other organs remained unchanged. Throughout the experiments the animals maintained high serum lithium levels of 1.3 to 1.8 mEq/1 on the average. These values are similar to those reported by Bera and Chatterjee (1976).

Biochemically, long-term administration of lithium carbonate under these conditions produced changes in the protein synthetic activities in two separate organs—the brain and the kidney. These results demonstrated that lithium's effects on the cellular protein synthesis in these two organs are different in nature. Individually, both brain and kidney ribosomes demonstrated different levels of biological activity, with brain ribosomes exhibiting higher incorporating activities relative to controls and kidney ribosomes exhibiting lower incorporating activities relative to controls. Ribosomes from both organs actively incorporated (^{14}C)leucine into protein for at least up to 60 minutes. These findings are highly interesting and should provide some understanding of the mechanism involved in the lithium action on brain, which to date is not clearly understood. Additional difficulties are again present, as the neurochemical basis of manic-depressive illness is also not well established. The present data also demonstrate that, at least in the brain, the lithium-induced changes in protein synthesis are dependent on the dosage of lithium administered to the animals. While biochemically, Li^+ exerts multiple effects on the metabolism of cerebral RNA, glutamic acid, carbohydrate, and uric acid, clinically, lithium is thought to have an uricosuric effect when it is used in the treatment of manic-depressive illness.

In the present report we have shown that Li^+ when given chronically can alter cellular protein synthesis, as measured by the incorporation of (^{14}C)leucine into nascent polypeptide chains in at least two tissues. The observed effects are organ specific, and although both organs are differentially affected by Li^+, the possibility exists that effects on kidney and brain may be partly coupled.

Anumonye et al. (1968) have postulated that Li^+ may interfere with the active transport of organic acids both in the kidney and in the brain. The present findings on Li^+ effects on the brain point to the increased biological activity of ribosomes as a result of direct action on a specific cellular constitutent, the ribosomes of the brain. Thus, one hypothesis will support the notion that in the brain the major action of Li^+ on RNA metabolism is mediated via protein synthesis. This conclusion is further substantiated by Anumonye et al. (1968), who showed parallel changes in mood in the manic-depressive patients occurring with changes in daily uric acid excretion. The finding of increased urinary output in our studies and elsewhere would signify not only a Li^+-induced uricosuric effect but also a major alteration in the purine metabolism of which uric acid is one of the major end products. The Li^+-induced stimulation of brain protein synthesis in our studies is further supported by the data of Dewar and Reading (1974) reporting on lithium-induced increased activities of Mg^{++}-dependent RNA polymerase. These authors further speculated that Li^+ effects on the kidney could be reflecting changes occurring in the cerebral RNA metabolism. These effects on brain RNA metabolism can also be directly correlated with present effects on Li^+-induced changes in protein synthesis. The findings on the Li^+-induced stimulation of in vitro brain protein synthesis are important in understanding the process of manic-depressive illness, as protein synthesis has been implicated in learning and memory processes (Glassman and Wilson 1970). Since cerebral protein synthesis is altered by chronic lithium treatment, one could speculate that the synthesis of specific proteins or of one protein could be altered. The alteration in synthesis of such a protein or proteins could be linked to modifications of mood as observed in the treatment of manic-depressives with lithium. One of the significant leads that arises from these studies focuses on the question of whether there is indeed an induction of a specific protein or proteins following lithium treatment in the brain. The synthesis of such proteins will have important implications in understanding manic-depressive illness.

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The observation on kidney ribosomes again provide some valuable information. The inhibitory effects observed in kidney protein synthesis in the present studies could be due to the nephrotoxic effects exerted by Li^+. Since uric acid is known to be the end product of purine metabolism, it is possible that it may also cause secretion of other acids into the cerebrospinal fluid (CSF). Guildberg et al. (1966) have shown that a mechanism for the transport of organic acids exists for the active removal of the acids from the brain and CSF into the blood with characteristics similar to that of the renal tubules mechanism for organic acids. On the basis of these data, we would hypothesize that (a) lithium exerts a primary effect on the process of protein synthesis in the brain; (b) Li^+ effects in this tissue are mediated via protein synthesis and are dose dependent; (c) these effects may involve the specific synthesis of some proteins with very special functions in the CNS, and these proteins may play a very specialized role in depression; and (d) the nephrotoxic effects of lithium reported in the literature are probably due to inhibition of protein synthesis in the kidney, which can be partly if not wholly triggered by alteration in brain chemicals. Therefore, the inhibition observed in kidney is related to the uricosuric effects of Li^+. In this respect, it is important to compare the effects of lithium with those of ethanol on brain protein synthesis. In contrast to Li^+, ethanol administration intragastrically or orally in drinking water inhibited brain protein synthesis (Tewari and Noble 1975; Tewari et al. 1980). Clinically, Li^+ is sometimes used in the treatment of alcoholism. Data presented in this report are thus of special significance, as Li^+ and ethanol exert opposite effects on the protein synthetic machinery of neural cells.

Summary

1. Effects of 6 weeks of intragastric intubation of 150 mg/kg or 225 mg/kg of lithium carbonate were determined in the in vitro incorporation of (^{14}C)leucine into protein by the brain and the kidney ribosomal systems.
2. Data showed increased body weight, polydipsia, and polyuria as early as 2 weeks following Li^+ intubation.

3. $Li^+$was found to have differential effects on the in vitro protein synthetic activities by the brain and kidney ribosomal systems.
4. Li^+ was found to be primarily inhibitory to kidney protein synthesis at both concentrations; the brain ribosomal system was stimulated to a greater extent, but only at the lower concentration of lithium. The observed stimulation was greatly reduced when the concentration of lithium was increased.
5. Data suggest that Li^+ action on protein synthesis is dependent on (a) the dosage of Li^+, (b) the specific functional property of the tissue, and possibly (c) the time period to which animals were exposed to Li^+.

Figure 1. Lithium Level in Serum of Rats

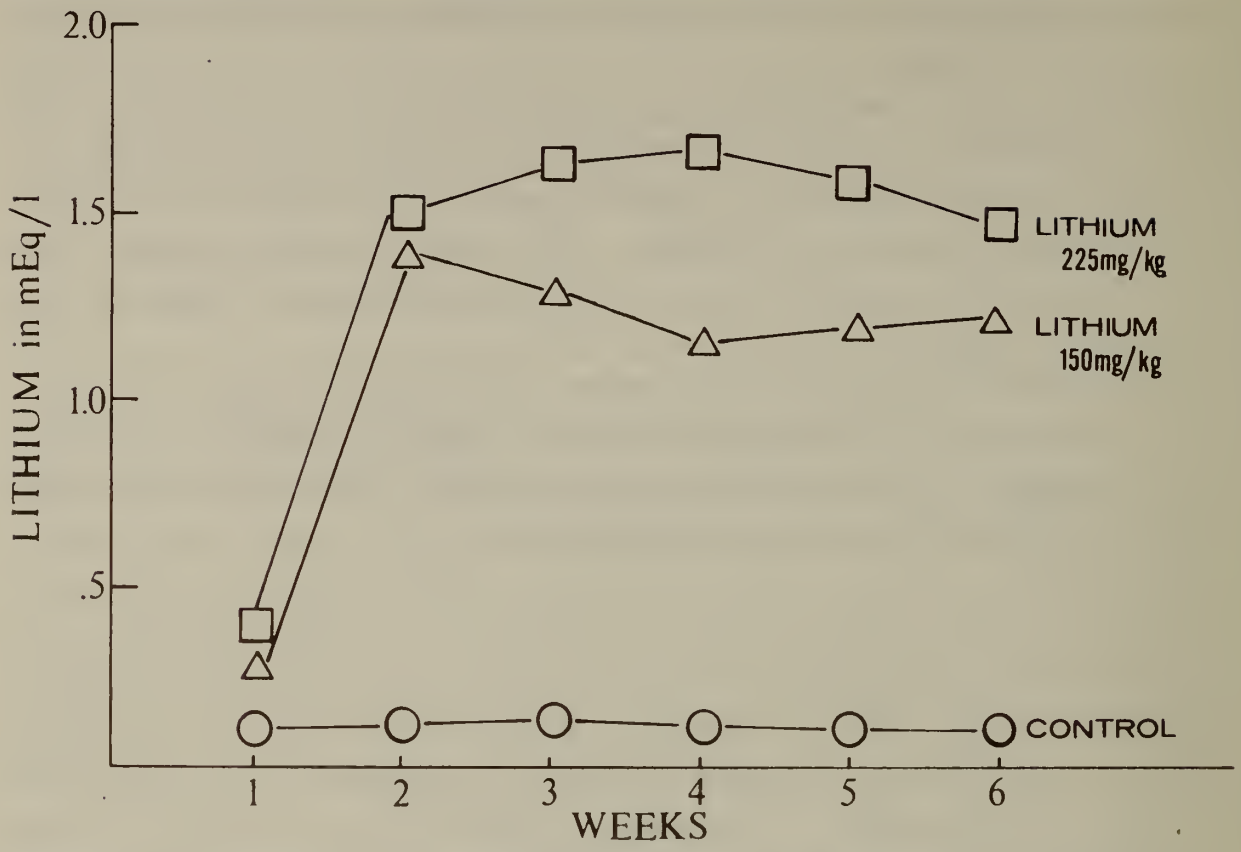

Figure 2. Effects of Lithium on the In Vitro Incorporation of (^{14}C)Leucine Into Protein by Rat Kidney Ribosomal System

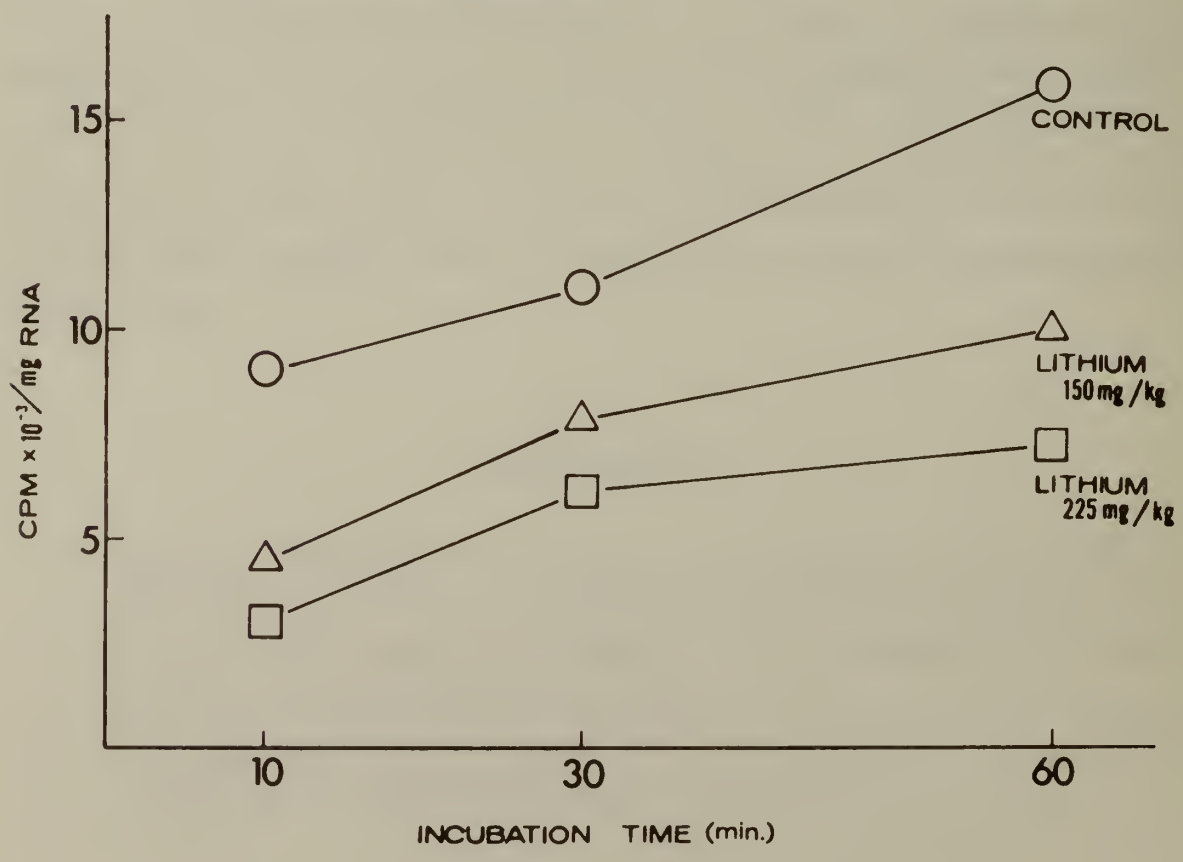

Figure 3. Effects of Lithium on the In Vitro Incorporation of (^{14}C)Leucine Into Protein by Rat Brain Ribosomal System

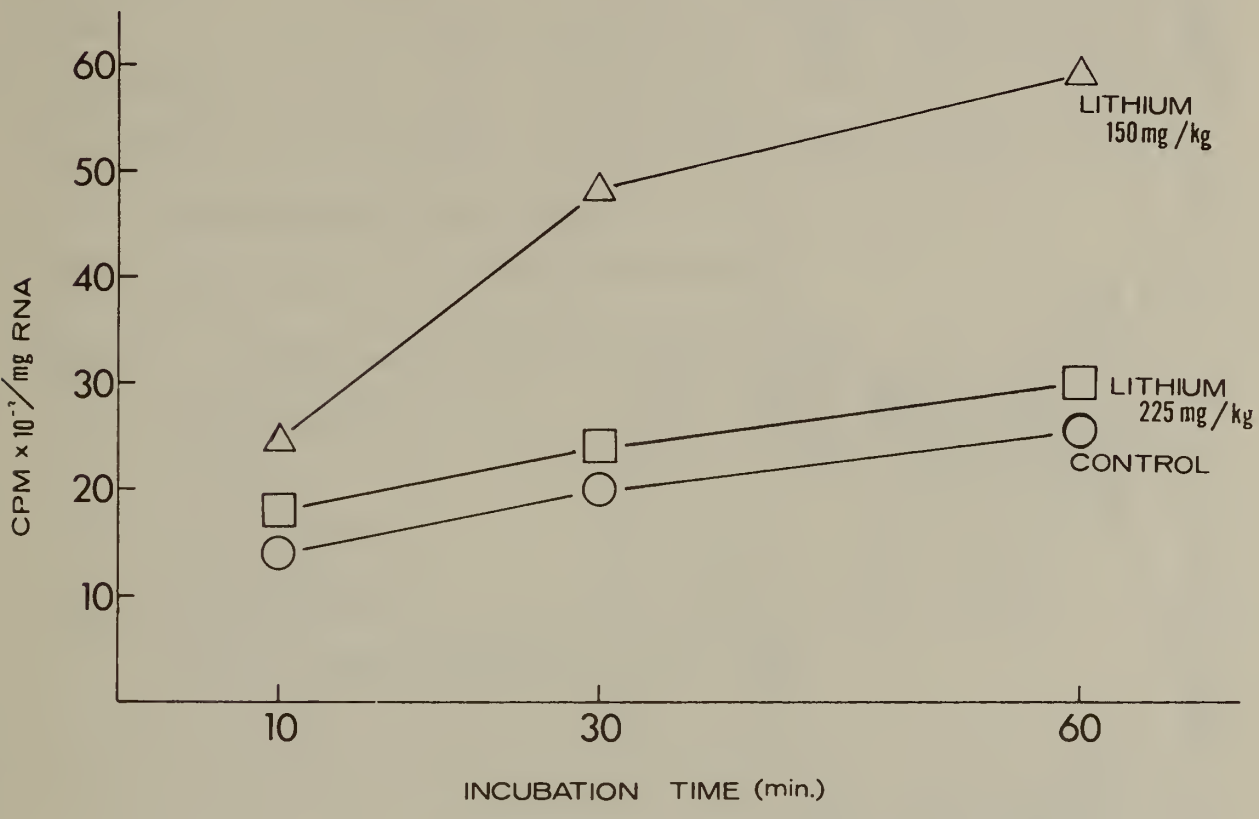

Table 1. Animal Treatment Conditions

Group	Number of Animals	Doses of Lithium Carbonate	Food
1	20	—	Granulated food
2	20	150 mg/kg daily	and water to drink,
3	20	225/kg daily	ad libitum

Note: The specific lithium treatment conditions have been described in the text.

Table 2. Effect of Chronic Lithium Carbonate Administration on the Body Weight, Water Ingestion, and Urine Output of Rats

Doses of Lithium Carbonate	Body Weight		Ingestion of Water per 24-hr period (mls/rat)	Urine Output per 24-hr period (mls/rat)
	Start of Study (gms/rat)	End of Study (gms/rat)		
0 (Control Group)	320 ± 20[a]	450 ± 30	41 ± 4	16 ± 4
150 mg/kg	320 ± 15	490 ± 50	115 ± 30	70 ± 20
Percent Control	100	109	280	438
225 mg/kg	295 ± 30	510 ± 35[b]	132 ± 50	95 ± 30
Percent Control	92	113	322	594

[a] Values represent mean ± SD.

[b] $P < .05$ when compared with control.

Table 3. Effect of Chronic Lithium Carbonate Administration on Organ Weight

Doses of Lithium Carbonate	Wet Weight of Tissue (gms/animal)			
	Brain	Liver*	Kidney	Testes
Control	2.1 ± 0.25	13.5 ± 1.9	2.56 ± 0.38	2.7 ± 0.43
150 mg/kg/day	2.4 ± 0.24	13.8 ± 1.4	2.84 ± 0.47	3.0 ± 0.42
225 mg/kg/day	2.4 ± 0.26	15.3 ± 1.3	3.06 ± 0.20	3.3 ± 0.60

Note: Values represent mean ± SD.

* These weights represent partial lobes of the liver.

Table 4. Lithium Level in Urine of Rats

Doses of Lithium	mEq/L	mEq/24 hr
Control	< 0.1	< 0.002
150 mg/kg/day	0.38 ± 11	3.12 ± 0.42
225 mg/kg/day	0.55 ± 16	5.18 ± 0.69

Note: Values represent mean ± SD.

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Evidence for the Psychogenetic Theory of Drug-Seeking Behavior

Kenneth Blum

The widespread abuse of opiates and ethanol in modern society has resulted in tremendous scientific investigation into the mechanisms of action of these two diverse classes of drugs. A review of the literature reveals increasing evidence for the support of the common mechanism theory of opiate and alcohol dependence. The basis of the theory resides in the potential of isoquinolines, byproducts of alcohol ingestion, to interact at opiate-mediated sites and serve as a "link" between alcohol and narcotics.

Recently, we proposed that alcohol- and opiate-seeking behavior is a function of endogenous peptidyl opiate levels and termed the theory "The Psychogenetics of Drug Seeking Behavior." A simple mathematical representation of the components of the theory is as follows:

$$DSB = G_{DIO} + E$$

where DSB = drug-seeking behavior, G_{DIO} = genetic deficiency of the internal opiate, and E = environment.

The theory proposes, in the case of alcoholism, three types as follows:

$$\text{Type I} = AD = G_{DIO} + E$$
$$\text{Type II} = AD = G_{NIO} + E$$
$$\text{Type III} = AD = G + {}_{NIO} + E + {}_{ADIO}$$

where AD = alcohol desire, DIO = deficiency of internal opiate, A = alcohol, G = genetic, E = environment, and NIO = normal internal opiate.

Evidence for Type I is derived from our finding that a negative correlation (R = 0.93) has been found between whole brain enkephalin levels and ethanol preference in DBA, C_3H, C-58, and C-57 mice. Currently, there is some experimental support for the Type II alcoholic from the earlier work of Masserman and Yum. Evidence for Type III is derived from our finding that a significant reduction in immunoreactive enkephalin-like material was observed in basal ganglia of hamsters consuming 10 percent ethanol on a 12-month preference schedule. Compared to paired controls, the latter work supports the negative feedback theory for agonist (opiate-like) induced inhibition of neuronal enkephalin synthesis as proposed by Goldstein. The psychogenetic theory of drug-seeking behavior serves as a useful model to investigate the underlying mechanisms involved in the phenomenology of substance and alcohol misuse. [Paper was not available for inclusion in the text of this Monograph.]

Ethanol Metabolism, Albumin Production, and Alcohol Dehydrogenase Activity During the Growth Cycle of Adult Rat Hepatocytes in Primary Culture*

Pushkaraj J. Lad, Harold Skelly, Bernard de Hemptinne, Katherine S. Koch, and Hyam L. Leffert

Abstract

Primary monolayer cultures of adult rat hepatocytes permit long-term regulatory studies under well-defined conditions. These cultures are proliferation competent, and they display developmental "programs" of differentiated function. The functions decline during the logarithmic phase and reappear as the cell population enters the stationary phase. When alcohol dehydrogenase activity and the rates of conversion of (^{14}C)ethanol into chloroform:methanol (2:1 v/v) extractable material were followed during the growth cycle, "U-shaped" curves were seen. (^{14}C)Ethanol conversion rates were ethanol concentration dependent; however, the addition of 0.1 to 1.0 mM pyrazole decreased these rates, but only at low ethanol concentrations (< 1 mM). Twelve-day-old cultures can be stimulated to proliferate (monitored by (^{3}H)thymidine uptake into DNA) by adding fresh growth media; the addition of 163 mM ethanol (749 mg%) to such cultures did not inhibit DNA synthesis, but markedly lowered stimulated (^{3}H)uridine and (^{3}H)leucine uptake into RNA and protein. The latter effects were ethanol concentration dependent, but were not seen in unstimulated cultures. Rates of total protein synthesis

NOTE: Figures and tables appear at end of paper.

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and the synthesis and secretion of albumin were studied in these hepatocyte cultures. These results are discussed in relation to ethanol's effects on cultures bearing different ADH levels.

To determine how ethanol causes physiological and pathological changes in the liver, previous investigators have studied ethanol's effects on intact animals, liver slices, and freshly isolated hepatocyte suspensions. Here we review a series of ethanol-related studies using proliferation-competent, long-term primary "monolayer" cultures of adult rat hepatocytes (Lad et al. submitted for publication 1981*a*,*b*; Leffert et al. 1980). Three fundamental areas of alcohol research were explored: (a) an analysis of alcohol dehydrogenase activity (ADH) and ethanol metabolism in the cultured hepatocytes, (b) the effects of "acute" ethanol treatments on cultured hepatocyte growth and function, and (c) proliferative and differentiated properties of cultured hepatocytes isolated from rats exposed chronically to intoxicating blood ethanol levels in comparison to similar cultures isolated from normal rats.

Properties of the Culture System

A typical growth curve of hepatocytes cultured in this laboratory is shown in figure 1. The hepatocytes are prepared routinely by a modified collagenase procedure. About 1×10^6 cells are plated into untreated plastic tissue culture dishes containing 2 ml of media lacking arginine to select for arginine-synthesizing cells (Leffert, Koch, Moran, and Williams 1979). Soon after plating, 20 to 30 percent of the cells clump and "flatten." Aggregates form within a few days (see Koch and Leffert 1980). DNA synthesis and cell division begin between days 2 and 4 without further medium changes and continue until days 8 through 12, after which a stationary phase is reached. Various kinds of evidence suggest that bulk growth results from hepatocyte proliferation (Koch and Leffert 1980; Leffert and Koch 1980).

A typical culture in early lag or stationary phases of the growth cycle displays many adult hepatocyte properties (Brown et al. 1980; Leffert et al. 1978). Among them are an intact urea cycle; a glucagon-responsive adenylate cyclase system; mixed function P_{450}-oxidase, glutathione S-transferase B, and pyruvate kinase type-L activity; and the capacity to convert lactate into glucose. During an in vitro growth cycle, hepatocytes show growth-state-dependent changes in most of these differentiated functions. The adult

phenotypes decline during logarithmic growth and reappear as the cell population enters a stationary phase (Leffert et al. 1978).

ADH and Ethanol Metabolism

Growth-cycle changes in ADH are shown in figure 2. The activity fell steadily during lag and early log phase (days 1 through 3). Activity was barely detected in cell homogenates prepared from 4- to 7-day-old cultures (log phase); it reappeared after day 8 and returned to initial levels as the population entered a stationary phase (day 12). ADH is expressed as units of activity per milligram of protein; therefore its decline after plating could not be due to cell death. Nor was ADH activity detected in the medium. This finding suggests that decreased activity is not due to enzyme leakage from hepatocytes. The "U-shaped" pattern of the curve eliminates the possibility that the decrease was caused by nutritional depletion, since at no time during the growth cycle were fresh media changes made (figures 1 and 2). Notably, ADH activity in fresh liver homogenates (shown as the disconnected point in figure 2) was similar to ADH activity in days 0 to 1 and day 13 cultures. More details of these studies are given elsewhere (Lad et al. submitted for publication 1981*a*; Leffert et al. 1980).

A similar U-shaped curve was observed for ethanol metabolism during the growth cycle. This was measured by following the incorporation of (^{14}C)ethanol metabolites into cellular lipid (figure 3). The metabolic activity measured by (^{14}C)ethanol conversion rates was determined from radioactivity recovered in a chloroform:methanol (2:1) extractable fraction per million cells (Lad et al. submitted for publication 1981*a*). It is worth noting that for these studies (and those mentioned below) cellular ethanol levels and the effective ethanol doses are unknown. Despite these problems, including the fact that ethanol evaporates from culture media, ethanol's cellular metabolism monitored by (^{14}C)precursor conversion is linear for a 12-hour duration under the conditions of these studies (Lad et al. submitted for publication 1981*a*).

(^{14}C)Ethanol conversion rates in day 10 through 12 hepatocyte cultures were more than tenfold higher than rates observed in 3T3 "fibroblast" cultures (Lad et al. submitted for publication 1981*a*). Parallel U-shaped ADH activity curves and (^{14}C)ethanol conversion rate curves suggest that ethanol metabolism measured radioisotopi-

cally, at initial extracellular concentrations ≤ 16 mM ethanol, is proportional to ADH activity.

To see if other alcohol-oxidizing pathways, like catalase in microsomal ethanol-oxidizing systems, were present, the effects of pyrazole, a competitive ADH inhibitor, were studied in day 13 cultures. The results are shown in table 1. At initial ethanol concentrations <1 mM, 1 mM pyrazole lowered ethanol metabolism significantly. At higher ethanol concentrations, increased ethanol conversion rates were seen. These observations suggested that non-ADH pathways contribute to ethanol metabolism at elevated substrate concentrations in cultured hepatocytes. In addition, the results of table 1 suggest that 24-hour pyrazole treatment *induces* formation and/or activation of the latter systems, because the dose response curve is "linearized" (see also Lad et al. submitted for publication 1981*a*). Still other variables seem operative, such as pyrazole metabolism per se, since with shorter drug incubation times (e.g., 4 hours) more efficient blockade of ethanol metabolism was seen (data not shown).

The capacity of 1 mM pyrazole to inhibit ethanol metabolism during the growth cycle is shown in table 2. Here, conversion of 1 mM (^{14}C)ethanol was followed for 4 hours. A U-shaped curve was again seen. Less blockade by pyrazole occurred in day 7 cultures compared to cultures from the early or late growth cycle phases. This finding suggests that U-shaped changes in ADH activity reflect changes in enzyme levels.

The decline and reappearance of ADH- and ethanol-metabolizing activity simulated in vivo developmental changes. For example, fetal and newborn rat livers that were growing rapidly contained low levels of ADH activity, whereas mature, "nongrowing" livers contained high ADH (Raiha et al. 1967; Rawat 1976).

Ethanol and Protein Synthesis and Secretion

The effects of "acute" ethanol exposure on protein synthesis and secretion by cultures in various growth cycle stages were studied. In these experiments, spent culture media were replaced with fresh media containing (^{3}H)leucine plus or minus ethanol. Then precursor uptake was followed into overall cellular and secreted proteins as well as into cellular and secreted albumin during the next 12 hours. Assay methods have been reported (Lad et al. submitted for

publication 1981*b*; Leffert et al. 1978, 1981) and further details will be published elsewhere (Leffert et al. in preparation).

Table 3 shows that 163 mM ethanol (the initial substrate concentration) did not lower (^{3}H)leucine incorporation significantly into total cellular protein or into cellular albumin in high-ADH-containing day 13 cultures. Similar results were obtained with respect to secreted albumin quantitated by radioimmunoassay. However, in day 7 cultures lacking ADH (figure 2), 163 mM ethanol reduced (^{3}H)leucine incorporation into total cellular protein 29 percent and into cellular albumin 77 percent. With respect to the secretion of total radiolabeled protein, only a 10 percent reduction was seen, whereas the secretion of radiolabeled albumin was inhibited 81 percent (table 4). These inhibitory effects depend on initial ethanol concentrations. No changes in phase microscopic cell morphology or in cell numbers/dish ("toxicity" parameters) were seen at this time (i.e., after 12 hours' incubation); nor was (^{3}H)leucine uptake into an acid-soluble fraction impaired (not shown).

Late stationary phase hepatocytes (11 to 13 days postplating) can be induced to *reinitiate* DNA synthesis and divide, monitored by (^{3}H)dT uptake into DNA and by radioautography, after adding fresh culture medium containing physiological combinations of insulin, glucagon, and epidermal growth factor (Koch and Leffert 1979). Studies summarized elsewhere suggest that these peptides (especially insulin and glucagon) are required for hepatocyte proliferaton in vivo (Leffert, Koch, Moran, and Rubalcava 1979).

Ethanol's effects on DNA and RNA synthesis under reinitiation conditions are shown in figure 4. The addition of 163 mM ethanol did not inhibit DNA synthesis in unstimulated (no media change) or stimulated cultures (media change plus peptides; figure 4, panel A). Inhibition of DNA synthesis was detected only at very high initial ethanol concentrations (about 500 mM; not shown). When RNA synthesis was studied by monitoring (^{3}H)uridine uptake into cold, acid-insoluble, alkali-sensitive material, 163 mM ethanol reduced precursor uptake in the stimulated but not in the unstimulated cultures (figure 4, panel B). The inserted panel in figure 4, panel B, shows a dose-dependent ethanol inhibition of RNA synthesis. As little as 16 mM ethanol is effective.

The findings shown in tables 3 and 4 and figure 4 show that ethanol acts directly on hepatocytes to inhibit different macromolecular processes at different ethanol threshold levels. The availability of ADH seems to be at least one determinant of cellular

"protection" to inhibitory effects of ethanol (and *not* a metabolite), although further work is needed to confirm this conclusion directly.

Cell Culture Studies With Hepatocytes Isolated From In Vivo Ethanol-Intoxicating Regimens

Proliferation and functional properties of hepatocyte cultures made from livers of chronically ethanol-intoxicated animals (8 to 12 days of blood ethanol levels of 210 mg%; Rogers et al. 1979) were compared to similar properties of cultures made from livers of untreated control animals. No differences in cell multiplication or in DNA synthesis rates were found (Lad et al. submitted for publication 1981*b*; Leffert et al. 1980). In addition, as shown in histograms of figure 5, panels A and B, stationary cultures from both experimental and control groups responded similarly to peptide proliferogens (Lad et al. submitted for publication 1981*b*).

However, differences in albumin secretion were observed, as shown in figures 6 and 7 (the sum of cellular and secreted data = total). When the incorporation of (^{3}H)leucine into immunoprecipitable albumin was studied (figure 6; Lad et al. submitted for publication 1981*b*; Leffert et al. 1978; Leffert et al. in preparation), cultures from alcoholic rats showed a significant late growth cycle reduction in the amounts of synthesized albumin released into the media (secreted fraction; figure 6, panel B). Because no intergroup differences were seen in the cellular levels of synthesized albumin (figure 6, panel A), it appears that this inhibition does not occur because albumin accumulates inside the cells. Similar results were obtained, and, in fact, greater reductions in secreted albumin were seen when the absolute amounts of albumin were determined directly by radioimmunoassay (figure 7, panels A and B). Moreover, these intergroup differents were not evident in cultures prepared from rats that were withdrawn from ethanol for more than 7 to 10 days (Lad et al. submitted for publication 1981*b*). These results suggest that a *specific* transient defect in albumin secretion is caused by chronic ethanol treatment. This conclusion is supported by additional observations because, unlike albumin, no intergroup growth cycle differences are evident with respect to (^{3}H)leucine incorporation into proteins precipitated by monospecific antisera to α_1-fetoprotein (figure 8).

The reasons why albumin secretory functions fail to reappear in liver cell cultures prepared from chronically ethanol-treated animals are unknown. Present evidence is consistent with at least two explanations. One involves defective redifferentiation of secretory components; the other involves defective redifferentiation of those molecules required for the synthesis of albumin that is destined for secretion. Further discussion of this problem appears elsewhere (Lad et al. submitted for publication 1981*b*; Leffert et al. 1980).

Summary

Proliferation-competent primary hepatocyte cultures metabolize ethanol and contain alcohol dehydrogenase activity. The culture system is useful for studying the regulation of ethanol metabolism and ethanol-metabolizing enzymes. In vitro changes in ethanol metabolism and in alcohol dehydrogenase activity appear to simulate in vivo developmental changes in similar specialized properties. Therefore, cultures in transition from low to high ethanol-metabolizing capacity may be useful not only to study ethanol's effects under these conditions but to delineate mechanisms that control "developmental programs" in hepatocytes as well.

The effects of ethanol on DNA, RNA, and protein synthesis are dose, time, and growth-state dependent. Liver cells cultured from chronically ethanol-treated rats display growth properties similar to cells cultured from normal rats. But during the stationary phase, cultures from chronically treated animals secrete less albumin compared to control cultures. The causes of this reversible redifferentiation "defect" appear to be specific for albumin (or, at least, adult export proteins), but the mechanisms involved are unknown.

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Figure 1. Adult Rat Hepatocyte Growth Curve in Primary "Monolayer" Culture

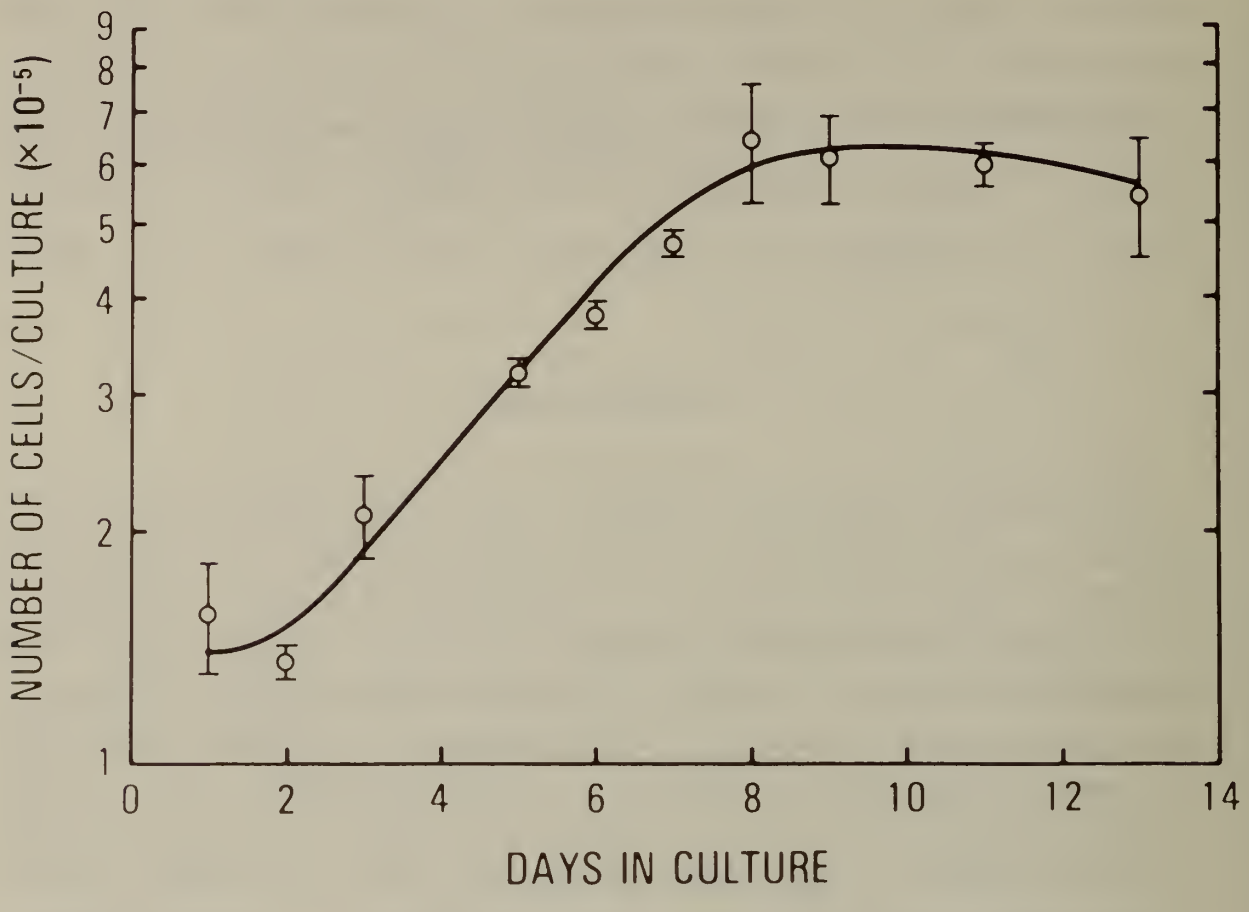
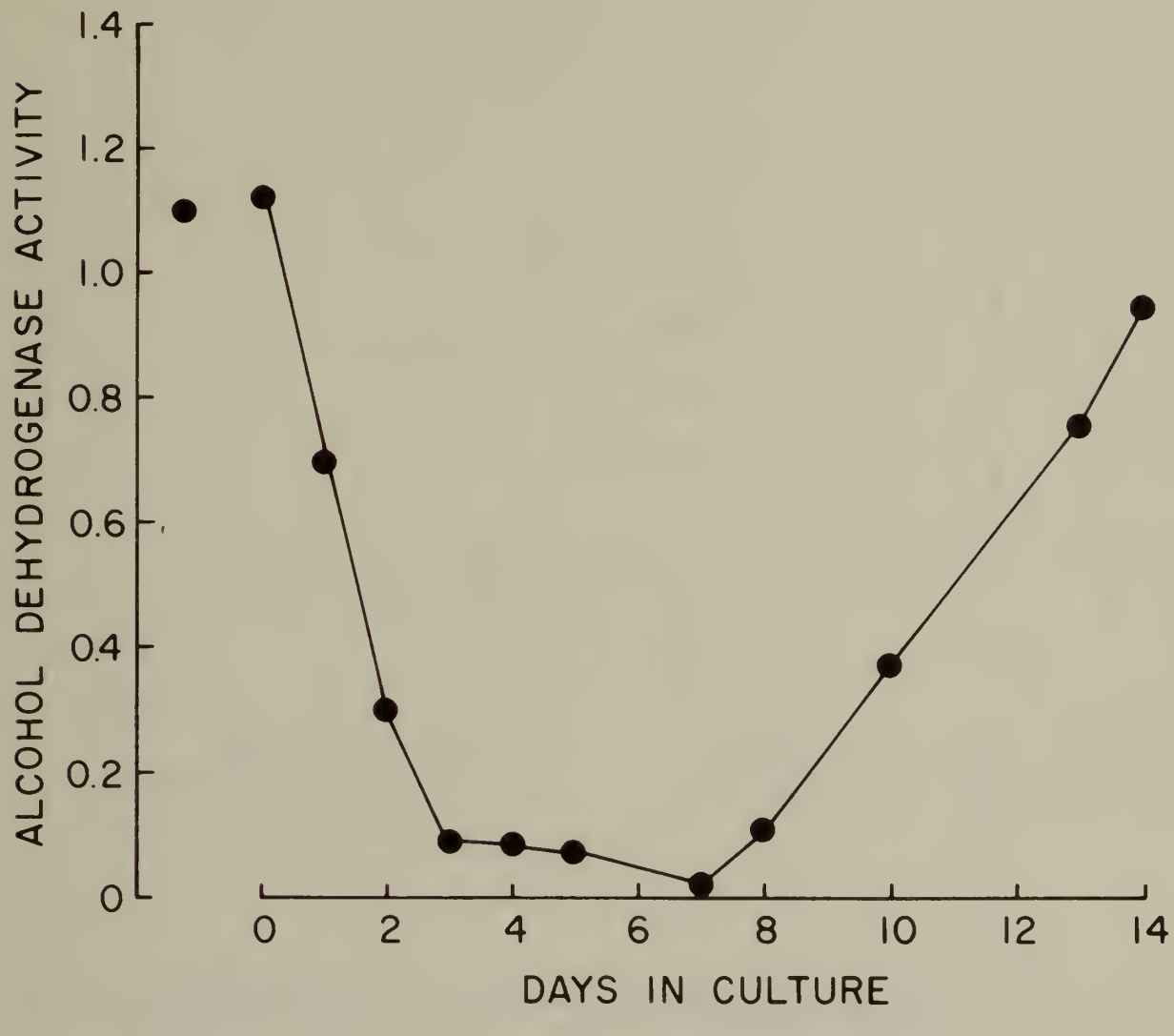
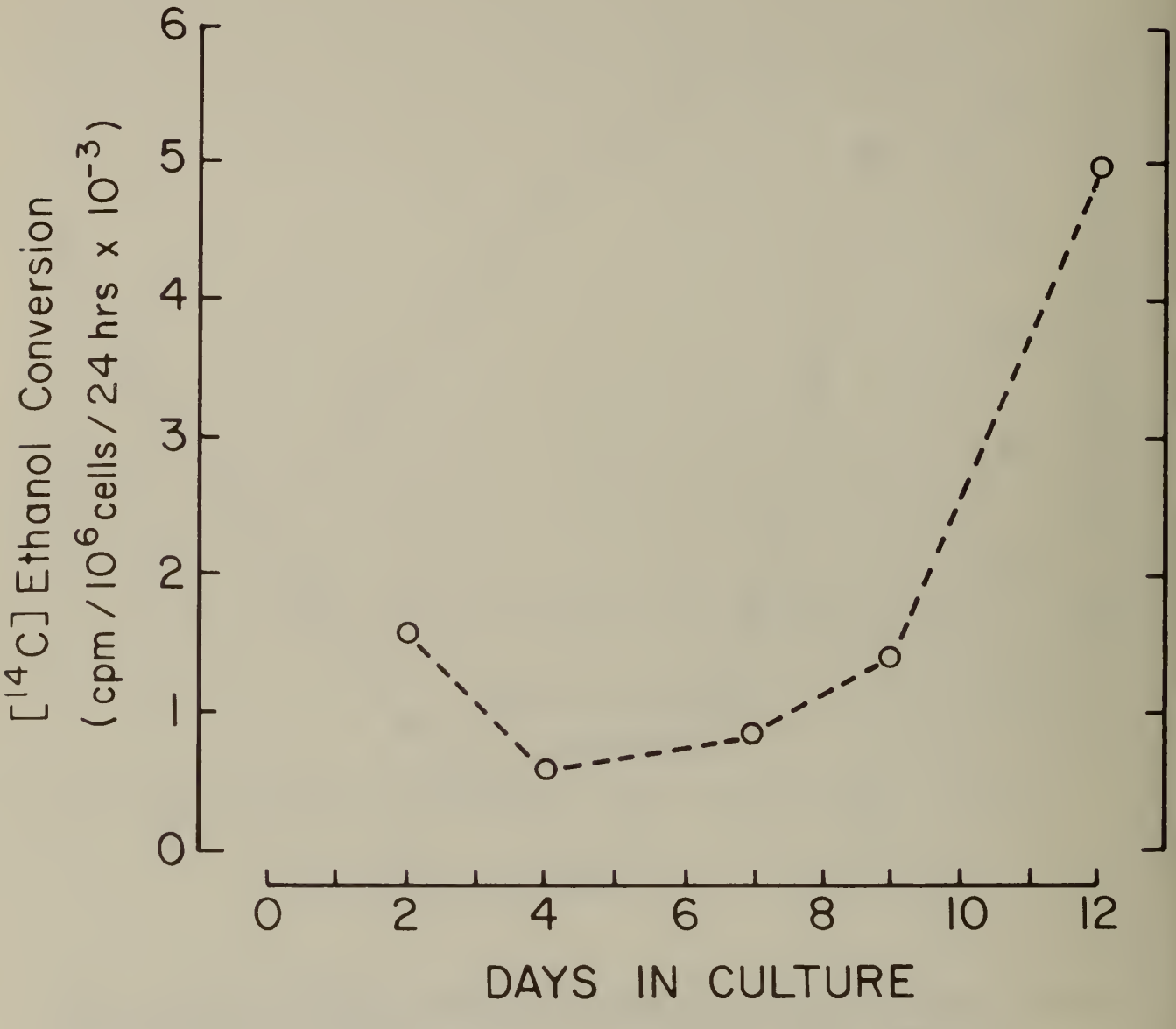


Figure 2. ADH Activity Levels During an In Vitro Growth Cycle of Adult Rat Hepatocytes



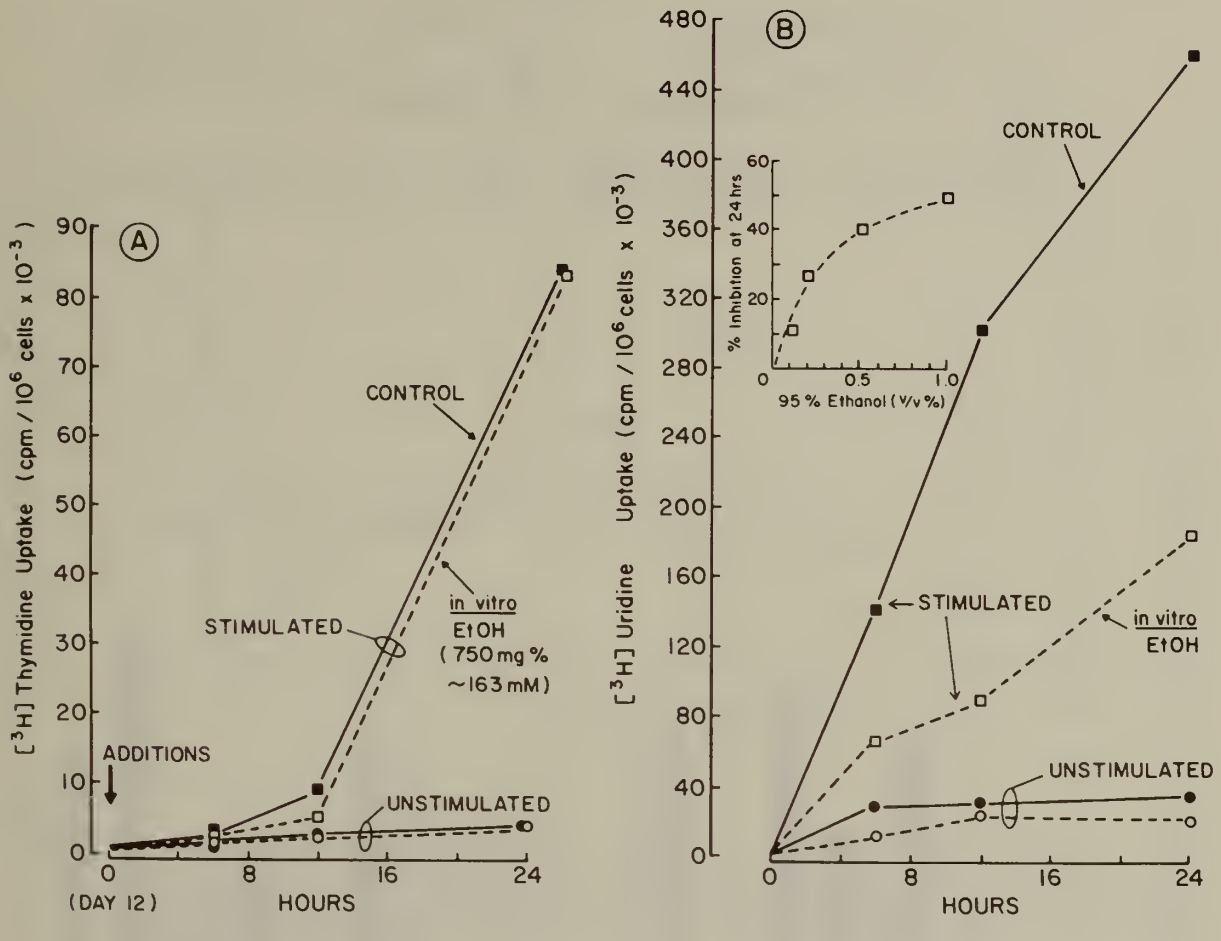
Note: Enzyme activity is expressed as nanomoles NADH formed per min per mg protein (Y-axis). For details, see Lad et al., submitted for publication 1981*a*.

Figure 3. Rates of (^{14}C)Ethanol Conversion Into Cellular Lipid During the Growth Cycle of Adult Rat Hepatocytes



Note: For details, see Lad et al., submitted for publication 1981*a*.

Figure 4. Effect of Ethanol on Hepatocyte DNA and RNA Synthesis in Day 12 Cultures



Note: Unstimulated cultures did not receive fresh media changes, whereas stimulated cultures received fresh media plus 50 ng each of insulin, glucagon, and epidermal growth factor (EGF) ml^{-1}. DNA (panel A) and RNA (panel B) synthesis were measured as described elsewhere (Koch and Leffert 1979). See note at table 1 for conversion of percent ethanol to mM ethanol (inset, panel B).

Figure 5. Growth-Reinitiation by Proliferogenic Peptides in 12-Day-Old Hepatocyte Cultures

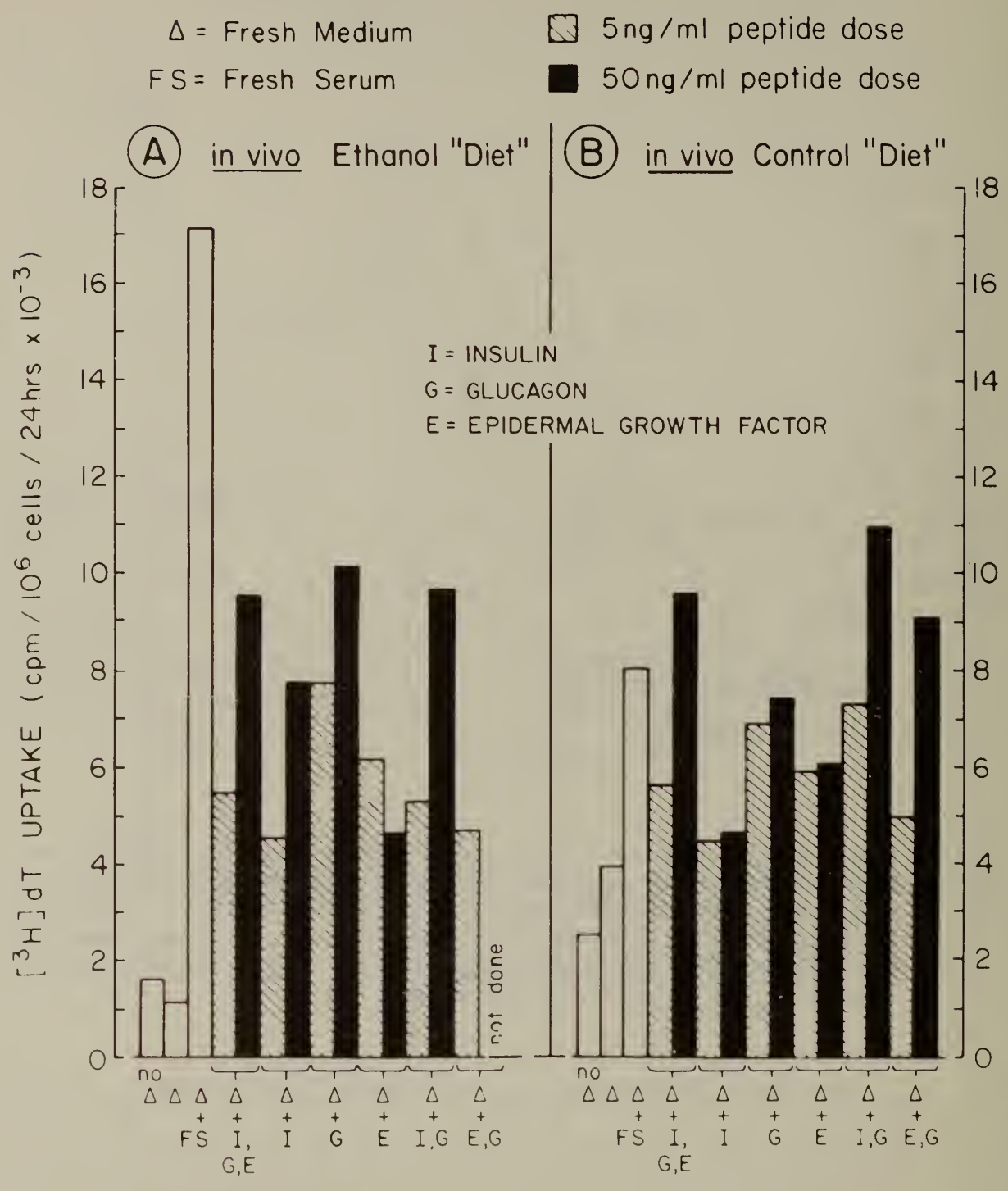
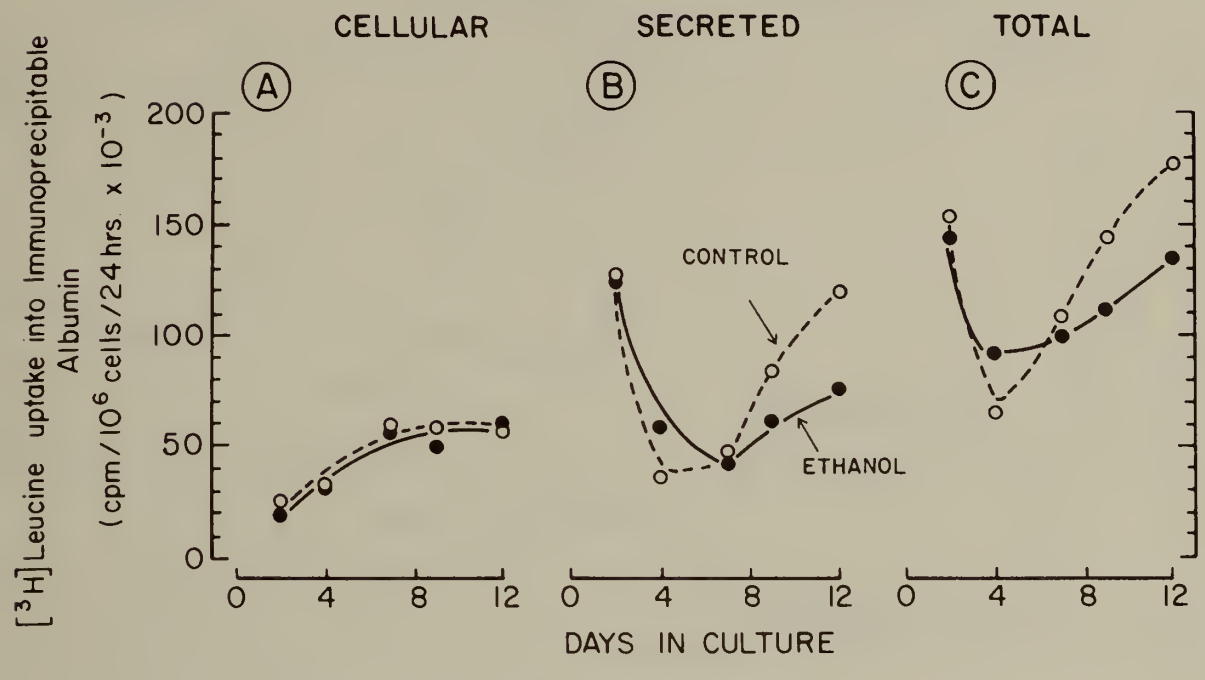
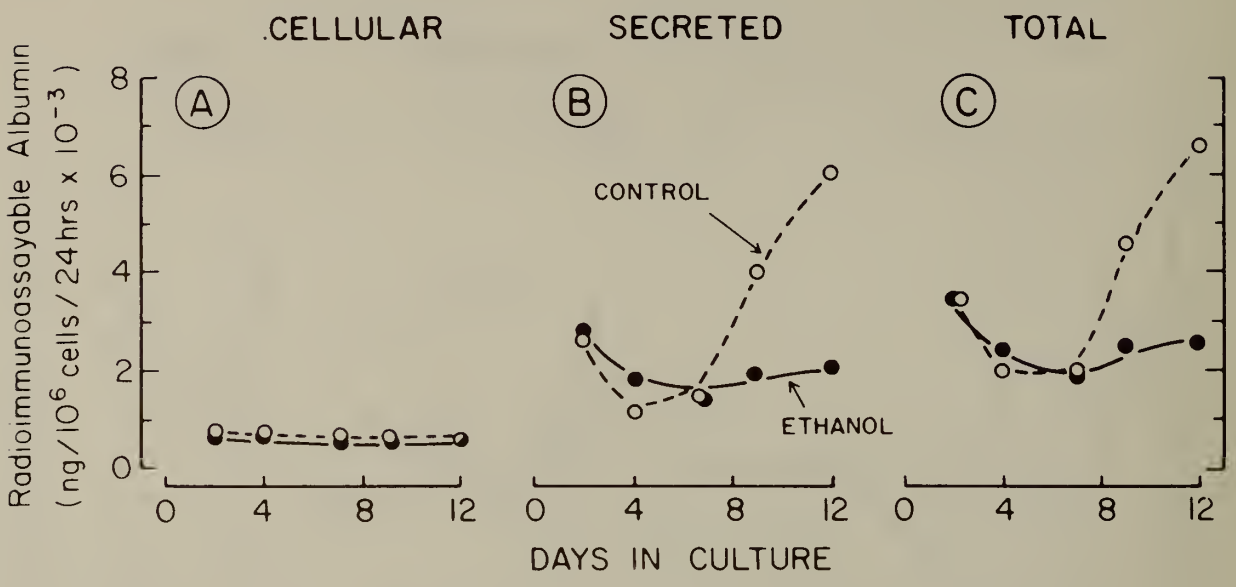


Figure 6. (^{3}H)Leucine Uptake Into Albumin Produced by Cultured Adult Rat Hepatocytes During a 12-Day Growth Cycle



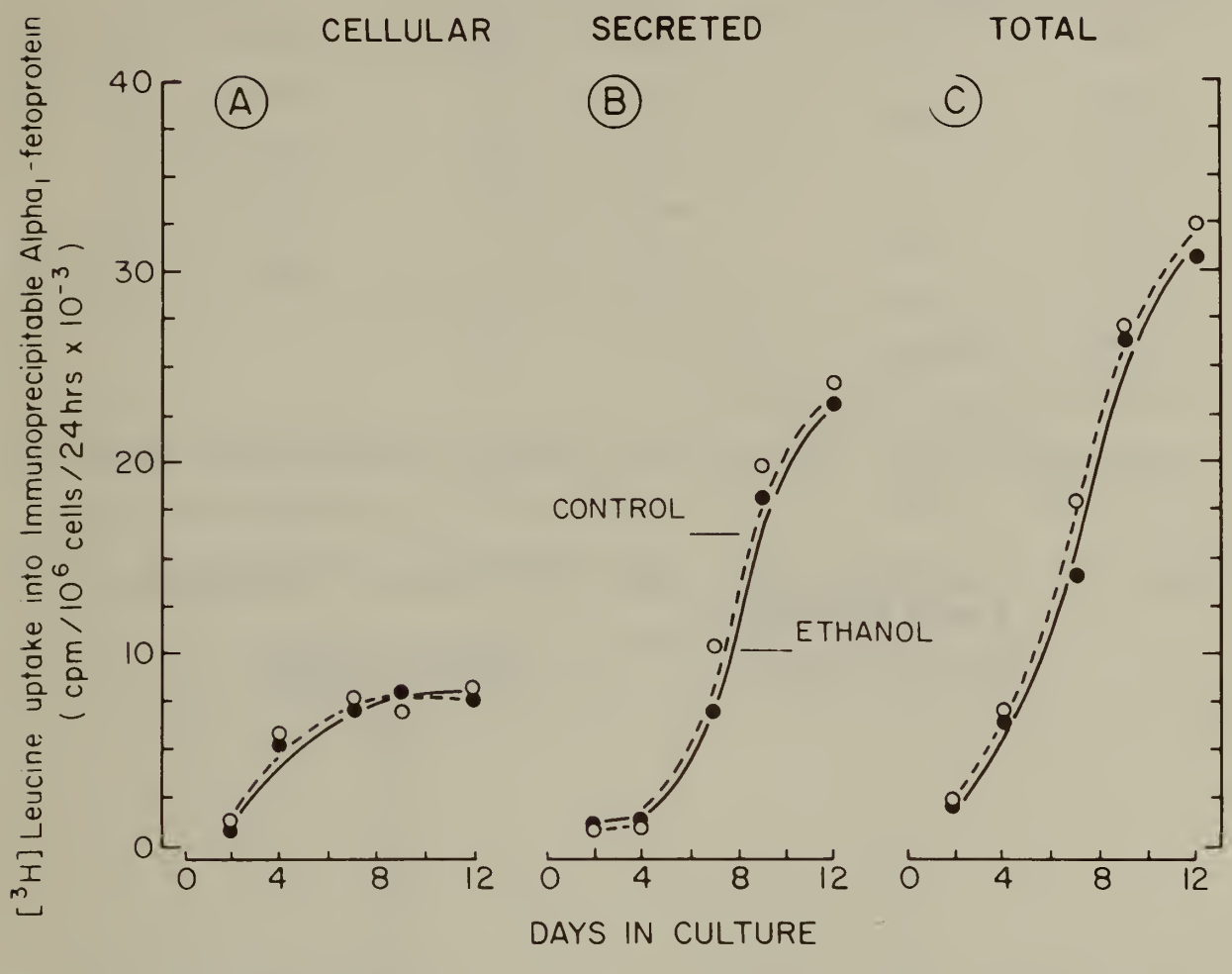
Note: Hepatocytes were obtained from chronically ethanol-intoxicated (solid lines) or mock-intoxicated control rats (dashed lines). Protein synthesis assays were performed as described by Leffert et al. (1978). For further details, see Lad et al., submitted for publication 1981*b*.

Figure 7. Radioimmunoassayable Albumin Produced by Cultured Adult Rat Hepatocytes During a 12-Day Growth Cycle



Note: See note to figure 6.

Figure 8. (^{3}H)Leucine Uptake Into Proteins, Immunoprecipitated by Monospecific Alpha$_1$-Fetoprotein Antisera, Produced by Cultured Adult Rat Hepatocytes During a 12-Day Growth Cycle



Note: See note to figure 6.

Table 1. Effect of Pyrazole on (^{14}C)Ethanol Metabolism in 13-Day-Old Cultures

Initial Ethanol Concentration (mM)	(^{14}C)Ethanol Conversion (nanomol/24 hr/10^6 cells)[a]	
	Control	Pyrazole (1 mM)
0.1	3.6 ± 1.2[b]	0.7 ± 0.4[b]
1.0	19.6 ± 3.0	16.6 ± 5.0
8.0	31.9 ± 3.4[b]	68.0 ± 10.0[b]
16.0[c]	48.2 ± 10.0[b]	92.3 ± 7.0[b]
50.0	108.0 ± 7.9	108.6 ± 4.8

[a] Values are averages ($N = 3$) ± SD.

[b] $p < 0.05$, control versus pyrazole.

[c] 16 mM ethanol is a 0.1 percent v/v solution of 95 percent ethyl alcohol in the culture medium.

Table 2. Inhibition of 1 mM (^{14}C)Ethanol Metabolism by 1 mM Pyrazole

Days After Plating	Percent Inhibition
2	48
5	32
7	9
10	37
13	76

Table 3. Effect of Acute Ethanol Treatment on Hepatocyte Protein Synthesis in 13-Day-Old Cultures

	Initial Ethanol Concentration (mM)	(^{3}H)Leucine Incorporation[a]		Albumin by Radioimmunoassay (ng/culture)
		Total Protein	Albumin	
Cellular	0	757.1	8.4	ND[b]
	163	745.8	8.0	ND
Secreted	0	1015.0	5.4	2,706
	163	976.0	5.3	2,990

[a] Leucine incorporation is expressed as cpm × 10^{-3}/culture/12 hr.

[b] Not determined.

Table 4. Effect of Acute Ethanol Treatment on Hepatocyte Protein Synthesis in 7-Day-Old Cultures

	Initial Ethanol Concentration (mM)	(^{3}H)Leucine Incorporation[a]	
		Total Protein	Albumin
Cellular	0	185.8	2.2
	163	132.2[b]	0.5[b]
	407	40.1[b]	0.6[b]
Secreted	0	133.7	2.6
	163	120.1	0.5[b]
	407	85.8[b]	0.2[b]

[a] Leucine incorporation is expressed as cpm $\times$ 10^{-3}/culture/12 hr.

[b] Values are significantly different from controls ($p < 0.05$).

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Brain Aminoacylation Reaction and Alcohol

Kevin B. Boylan and Sujata Tewari

Previous observations have reported ethanol-induced inhibition of in vitro protein synthesis in a brain ribosomal system. Additional studies observed early effects of ethanol on the initial steps of protein biosynthesis involving the formation of aminoacyl tRNA. These studies were further extended to determine effects of the development of physical dependence upon ethanol on the synthesis of (^{14}C)leucine tRNA. The in vitro formation of (^{14}C)leucine tRNA was measured in brains of rats consuming a nutritionally balanced liquid diet containing 36 percent ethanol-derived calories. Ethanol was withdrawn from half of the ethanol-ingesting group for 24 hours and replaced with control diet. Experiments were carried out following purification of brain enzymes from the control, the ethanol-dependent, and the withdrawn groups. Incubations were carried out in the presence of purified brain tRNA or yeast tRNA, (^{14}C)leucine, ATP, Mg^{++}, and necessary salts at 37 percent. Yeast tRNA was used as a standard source of tRNA for all three groups of enzymes. Results showed that the charging of tRNA from brain or yeast cells was linear for 20 minutes by enzymes from the three groups. All groups charged yeast tRNA successfully with (^{14}C)leucine. In addition, the in vitro formation of (^{14}C)leucine tRNA was dependent upon the concentration of tRNA present in the incubation media. When the tRNA to synthetase ratio was 0.2, data showed that the in vitro incorporation of (^{14}C)leucine into yeast tRNA was significantly reduced in the ethanol withdrawn group, although no appreciable differences were observed in the enzymatic activity between control and "ethanol" sources. However, with an increase in the tRNA/synthetase ratio to 2.0 again using yeast

tRNA, the formation of (^{14}C)leucyl-tRNA was considerably reduced in the ethanol-dependent group to the same extent as the withdrawn group. The control leucine tRNA synthetase activity was further measured in the presence of very low concentrations of brain tRNA from the control and experimental groups in an experiment where the tRNA/synthetase ratio was 0.02. Data showed that with the ratio of 0.02, the synthesis of (^{14}C)leucine tRNA by "control" synthetase was optimum when the source of tRNA was from the "withdrawal" group, with the "ethanol" tRNA also producing higher activity than the control. Somewhat similar results were obtained when the aminoacylation reaction was carried out using the 0.02 tRNA/synthetase ratio for all three groups. Data showed that in the control brain the aminoacylation was more than doubled when the tRNA/synthetase ratio was 2.0 or more. Although the high ratio increased the leucyl-tRNA synthesis in the experimental groups, the increase was not as substantial as that observed in the "control" group. The inhibition by ethanol was progressive when the tRNA/synthetase ratio was increased from $0.02 > 0.2 > 2.0$. The data suggest that the brain enzymes from ethanol-treated animals may be defective in charging high levels of tRNA when compared to controls. Experiments are in progress to further elucidate this property. [Paper was not available for inclusion in the text of this Monograph.]

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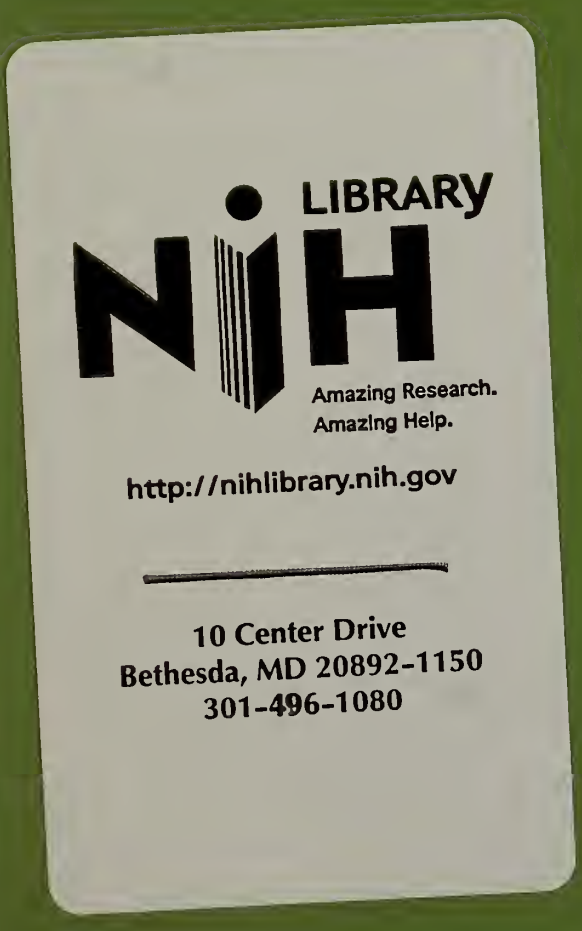

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